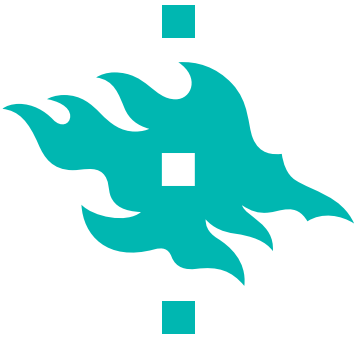

DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

SHIRIN TAVAKOLI

INTRAVITREAL LIPOSOMES AS OCULAR DRUG DELIVERY SYSTEMS: VITREAL INTERACTIONS, RETINAL PERMEATION AND DRUG RELEASE CHARACTERISTICS

DRUG RESEARCH PROGRAM
DIVISION OF PHARMACEUTICAL BIOSCIENCES
FACULTY OF PHARMACY
DOCTORAL PROGRAMME IN DRUG RESEARCH
UNIVERSITY OF HELSINKI

Drug Research Program
Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki
Finland

Intravitreal liposomes as ocular drug delivery systems: vitreal interactions, retinal permeation and drug release characteristics

Shirin Tavakoli

DOCTORAL DISSERTATION

To be presented for public examination, with the permission of the Faculty of Pharmacy of the University of Helsinki, in Lecture Hall 1041, Viikki Campus, Biocenter 2, on the 23rd of June, 2021 at 10 o'clock

© Shirin Tavakoli 2021

ISBN 978-951-51-7351-5 (pbk.)

ISBN 978-951-51-7352-2 (PDF)

Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis

ISSN: 2342-3161 (print) and 2342-317X (online)

The Faculty of Pharmacy uses the Urkund system (plagiarism detection) to examine all doctoral dissertations.

Hansaprint Oy,
Helsinki 2021

Supervisors: Professor Arto Urtti, Ph.D.
Drug Research Program
Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki
Helsinki, Finland

Docent Marika Ruponen, Ph.D.
School of Pharmacy
Faculty of Health Sciences
University of Eastern Finland
Kuopio, Finland

Professor Stefaan de Smedt, Ph.D.
Laboratory of General Biochemistry and Physical Pharmacy
Faculty of Pharmaceutical Sciences
Ghent University
Ghent, Belgium

Senior Researcher Eva M. del Amo Páez, Ph.D.
School of Pharmacy
Faculty of Health Sciences
University of Eastern Finland
Kuopio, Finland

Reviewers: Professor Elias Fattal, Ph.D.
Institute Galien Paris-Saclay
Faculty of Pharmacy
University Paris-Sud (Paris-Saclay)
Paris, France

Professor Steve Brocchini, Ph.D.
School of Pharmacy
University College London
London, UK

Opponent: Associate Professor Ilva Rupenthal, Ph.D.
Department of Ophthalmology
New Zealand National Eye Centre
Faculty of Medical and Health Sciences
The University of Auckland
Auckland, New Zealand

ABSTRACT

The prevalence of vision-threatening diseases of the posterior eye segment, such as age-related macular degeneration (AMD), diabetic retinopathy, diabetic macular edema and glaucoma, is increasing worldwide. This is a major burden to patients and health care systems. Current therapy includes intravitreal injections of anti-angiogenic agents against vascular endothelial growth factor (VEGF), because drug delivery to the back of the eye is hampered by anatomical and physiological barriers. Intravitreal injections are uncomfortable, may cause adverse reactions and reduced compliance leading to suboptimal treatment outcomes. Furthermore, current anti-VEGF drugs are not effective in all patients. Therefore, new drugs and delivery systems for targeted and prolonged action are needed for posterior segment eye treatment. Nanoparticles have been investigated as a long-acting and targeted ocular drug delivery systems that may prolong actions of small molecule drugs (e.g. corticosteroids and tyrosine kinase inhibitors) with short vitreal half-lives. Nanoparticles are also important for delivery of labile therapeutics with intracellular targets, including some proteins, neuroprotective peptides and nucleic acids (RNA, DNA). However, several barriers hamper retinal delivery of intravitreal nanoparticles and interspecies differences may lead to poor clinical translation. Hence, the overall objective of this study was to generate improved understanding of ocular barriers to retinal delivery of nanoparticles by using translationally valid preclinical models. We systematically studied vitreal diffusion of various liposomes and other lipid-based nanoparticles by analyzing the mobility of the nanoparticles with single particle tracking in intact porcine central vitreous with similar structure to human vitreous. We evaluated the physicochemical features of nanoparticles affecting their vitreal mobility (e.g. particle size, surface change, surface coating with polyethylene glycol or hyaluronic acid). Neutral and anionic liposomes showed faster diffusion than cationic liposomes. Small size and polymer coating modestly facilitated vitreal mobility of liposomes. Kinetic analysis demonstrated that nanoparticles' distribution in the human vitreous is controlled by convection rather than diffusion, while vitreous liquefaction may increase the role of nanoparticle diffusion. We also studied protein corona formation on liposomes' surface, since it may affect the hydrodynamic diameter and cellular interactions. In this regard, surface plasmon resonance analysis was performed to monitor the protein corona formation in the presence of porcine

vitreous. Insignificant size change was seen indicating that vitreal diffusion is not influenced by protein corona. In addition, high-resolution proteomics confirmed identity of the proteins on liposomal surface that may change the biological interactions of the liposomes. Next, retinal permeation of liposomes was studied systematically using *ex vivo* analyses and bovine retinal explants. Neutral and anionic liposomes with high vitreal mobility were studied for their potential in overcoming the ILM barrier. Liposomes with diameters over 100 nm fail in retinal entry irrespective of their surface charge, while small anionic PEG-coated liposomes (<50 nm) distributed into the retina. Lastly, a liposomal formulation was developed to encapsulate sunitinib, a small molecule anti-neovascular drug for VEGF suppression. Unlike sunitinib solution, the liposomal formulation showed anti-neovascular effect in laser induced mouse choroidal neovascularization model. In summary, this study extended understanding of the retinal drug delivery barriers related to the intravitreal injections. It also informed about the role of nanoparticles' characteristics on their interactions with ocular barriers. These findings can be leveraged in understanding pharmacokinetics and design of retinal drug delivery systems.

Acknowledgements

The work in this thesis project was carried out at the Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki during the years 2016-2021. This work was financially supported by the European Union's Horizon 2020 research and innovation program Marie Skłodowska-Curie Innovative Training Networks (ITN). The Phospholipid Research Center, Finnish Pharmaceutical Society and Sokeain Ystävät are gratefully acknowledged. Doctoral Program in Drug Research (DPDR) and Doctoral School in Health Sciences are also acknowledged for their support and for providing opportunities to participate in a variety of courses.

I would like to express my warm gratitude to all colleagues, family and friends who had a positive impact during the years of work on this thesis to be accomplished.

Special thanks to:

My supervisor *Prof. Arto Urtti*, who has supported and mentored me throughout my doctoral studies. Your expertise in the field of ocular drug delivery and pharmacokinetics, the way of your teaching, and your ideas have always inspired me and of course, provided a valuable perspective on my work. I appreciate your wisdom, relaxed and kind attitude, patience, understanding and optimism which made such a positive and encouraging atmosphere to grow as a researcher.

My supervisors at University of Eastern Finland, *Dr. Marika Ruponen* and *Dr. Eva del Amo* for your advice, time and support in any way, whenever I have needed it. I would like also to thank *Prof. Stefaan de Smedt* and *Prof. Katrien Remaut* at Ghent University for inviting me into your research group. I am grateful for your supervision and kind support during both of my research visits.

Prof. Ilva Rupenthal from the University of Auckland, New Zealand for accepting to be my opponent at the public examination of the thesis. I would also like to acknowledge my pre-examiners *Prof. Elias Fattal* from University Paris-Sud, France and *Prof. Steve Brocchini* from University College London, UK for their critical review and helpful comments on the thesis. I am grateful to my thesis committee *Dr. Heidi Kidron*, *Prof. Kai Kaarniranta* for their time and advice. In addition, I would like to extend my gratitude to *Prof. Mikko Airavaara* and *Prof. Timo Laaksonen* for accepting the responsibility to serve as grading committee of my thesis. *Prof. Marjo Yliperttula* for her kind support in post-graduate studies as the head of Biopharmaceutics discipline. *Prof. Jan van Hest* for his continuous support and excellent meetings during NANOMED project.

Prof. Molly Stevens for her support which allowed experiencing the exciting research with new analytical and imaging techniques during my research visit at Imperial College London. I would also like to thank *Prof. Koen Raemdonck* for having me in his research group and supported my second visit at Ghent University. I am grateful for his guide through the interesting CADs project. *Dr. Rien de Ruiter* and *Prof. Michel Eppink* who welcomed and supported me during my research visit at Synthon Company. *Prof. Ville*

Paavilainen and *Dr. Shahid Rehan* for your time and kind support with SEC analysis, even though this collaboration did not result to a publication.

Dr. Tatu Lajunen, my ex-mentor and supervisor-to-be in the light-activated liposome project. I enjoyed the work we did together and the interesting discussions we had. *Dr. Karen Peynshaert* for teaching me to develop the organotypic model of the retina. Thank you for a great deal of energy, wisdom and positivity you brought into the work we did together. I will never forget those mornings we drove to the slaughterhouse, our chitchats on the road and the amazing evening at Gentse Feesten! *Dr. Astrid Subrizi* for her generous time and support. You were my source of inspiration in scientific writing. *Dr. Otto Kari* for introducing me to the world of protein corona/proteomics and being an encouraging co-author with exciting ideas. Thanks also for your friendship, being an ear to listen, supports and advice.

My other co-authors, *Dr. Elisa Toropainen*, *Prof. Harri Alenius*, *Dr. Tapani Viitala*, *Dr. Joseph Ndika*, *Dr. Petteri Parkkila*, *Dr. Fumitaka Tasaka*, *Dr. Joke Devoldere*, *Jooseppi Puranen*, *Niklas Johansson*, *Mecki Schmitt*, *Julia Lehtinen*, *Tiina Turunen*, *Simone Baan*, *Roosa Savolainen* and *Teemu Ruoslahti* for their valuable contributions to this thesis.

To the colleagues and friends at UEF; *Dr. Veli-Pekka Ranta* for the interesting practical pharmacokinetic course, *Dr. Mika Reinisalo* and *Amir Sadeghi* for the collaboration and your kind support with everything I needed help with and *Marko Lamminsalo* for interesting discussions and your tips that helped me to receive my first research grant! To the colleagues and friends at Ghent University; *Joke*, *Silke*, *Mike*, *Cristina M.*, *Toon*, *Herlinde*, *Laurens*, *Heleen*, *Juan*, *Maryam* and *Elnaz* among others for making me feel welcome and made my research visit a wonderful experience. In addition, a big thank to *Karen*, *Jelter*, *Thijs* and *Toon* for the after-work Badminton evenings! Especially, *Molood* and her lovely mom, who always warmly welcomed and supported me during my stay in Ghent. Thanks for nourishing me with Fesenjoon, Ghalieh and Nabat;)! *Roberta* such a supportive and cheerful companion who also helped me with experiments and visualizing my research. *Cristina*, who completed our quartet in Ghent! Thanks for your friendship, the wonderful memories and the laughter. That last evening will stay with me forever! To my colleague at Imperial College London, *Dr. Jelle Penders* for his friendship and valuable support and help throughout the experiments with SPARTA and FIB-SEM. My other colleagues in NANOMED consortium, *Shoupeng, Mona, Roxane, Esra, Evangelos, Jerry, Mahsa, Conor and Jaleesa* for the great collaboration and friendship.

Wonderful people whom I had the honor to meet during my study as doctoral student. Thanks for making such an enjoyable working environment. Especially, *Ansku* whom I met the first day of my research and taught me how to dissect the eyes! Thanks for your friendship and kind support even after you left Finland. Also, thanks for welcoming me into the very early Mid-summer camp at Nuuksio and your travel company to several conferences, among which I think we will never forget the ARVO in Vancouver! Instead of reminiscing the less digested aspects of our trip, I fondly look back at the unforgettable time I had the stomach for overcoming my fear of heights and walked on the suspension Capilano Bridge! *Madhu* and *Eva* for your kind friendship, support and nice times we had

at work as well as outside the office. *Jaakko* for introducing me to macromolecules, mentoring me in the protein lab and also translating my grant application's abstract to Finnish! Thanks for being such an amazing fun friend and collaborator. *Vijay* for your kind help, interesting discussions, friendship and your company in our trips for the project meetings. Paulina for your friendship, laughs and fun moments we had at work and outside research. I also wish to thank (in no specific order) *Jacopo Z.*, *Feng*, *Teemu*, *Jacopo C.*, *Mariia*, *Eija*, *Patrick*, *Ali*, *Niki*, *Sami*, *Marja*, *Aniket*, *Nazanin*, *Riky*, *Riina*, *Sanjay*, *Noora*, *Wilma*, *Alli*, *Jasmi*, *Vili*, *Arto*, *João*, *Anusha*, *Anam*, *Victor*, *Cris*, *Manlio*, *Sara*, *Firas*, *Bea* and many more past and present members of the division of Pharmaceutical Biosciences. *Leena Pietilä* and *Lea Pirskanen* for their valuable help with the work. I would also like to thank *Timo Oksanen* for his time and help in UPLC analysis. *Kristoffer Nyström* and *Meeri Salmela* for their support at Biocenter 2.

I wish also to acknowledge maestro *Keyhan Kalhor* for his magnificent Setar Solo performance (Abgineh, Tehran) with which I wrote the whole thesis. It should have been replayed more than 300 times!

My closest friends outside research, *Rezvan* and *Mohammad* for the unconditional friendship and all wonderful memories we created together. *Faraz*, for his kind support and friendship. *Homa* and *Ali*, such kind-hearted and supportive friends. Thanks also for all the late-night board games from Evolution to K2 and Pandemic! *Vahideh* and *Mohsen* for their warm friendship and also their company in extreme Finnish adventures, especially, the Avanto swimming at -15 °C! *Ella* and *Jan* for great get-togethers and Mökki experiences.

My in laws, especially warmest thanks to my mother-in-law for her kind supports over these years. I also wish to thank my extended family for their warm welcome and the great weekends we spent together in London during my research visit.

My Brothers and sisters-in-law; *Shahram* and *Katy* for your unconditional support and kindness in general. *Shahram*, thanks for the perspective you provided on my personal life over our discussions on philosophy of life. *Bahram* and *Azita* for always being there and also the amazing discussions and time we had together every time I visited Delft. *Bahram*, thanks for your continuous support especially the first weeks that I moved to Finland, your presence, thoughtful advice and helps have been meant a lot. You all have been and still are my source of inspiration in different ways. *Dena*, the sunshine of the family! Thanks for the joy and energy you always share.

My wonderful parents to whom I cannot thank enough for their love, support, devotion, modesty, wisdom and patience. Without your help and encouragement, I would not have been able to make it as far as I have. Mamnoon Baba! Merci Maman!

Finally, I would like to express my deepest gratitude to *Sina*, my husband; ex-classmate back in Pharmacy school, friend, collaborator and co-author! You always stood by me during all challenges and made it possible to enjoy bittersweet moments of life. Thanks for your loving support, patience and being the constant source of encouragement in our years together.

Helsinki, June 2021
Shirin Tavakoli

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Tavakoli * S.**, Kari * O.K., Turunen T., Lajunen T., Schmitt M., Lehtinen J., Tasaka F., Parkkila P., Ndika J., Viitala T. and Alenius H., Urtti A., Subrizi A.: Diffusion and protein corona formation of lipid-based nanoparticles in vitreous humor: Profiling and pharmacokinetic considerations. *Molecular Pharmaceutics* 18 (2): 699-713, 2020.

II Kari O.K., **Tavakoli S.**, Parkkila P., Baan S., Savolainen R., Ruoslahti T., Johansson N.G., Ndika J., Alenius H., Viitala T., Urtti A., Lajunen T.: Light-activated liposomes coated with hyaluronic acid as a potential drug delivery system. *Pharmaceutics* 12(8): 763, 2020.

III **Tavakoli S.**, Peynshaert K., Lajunen T., Devoldere J., del Amo E., Ruponen M., De Smedt S., Remaut K., Urtti A.: Ocular barriers to retinal delivery of intravitreal liposomes: Impact of vitreoretinal interface. *Journal of Controlled Release* 328: 952-961, 2020.

IV **Tavakoli S.**, Puranen J., Bahrpeyma S., Lajunen T., Tropainen E., del Amo E., Ruponen M., Urtti A.: Liposomal sunitinib in ocular drug delivery: A potential treatment for choroidal neovascularization (Manuscript)

* Equal contribution

The publications and unpublished material are referred to in the text using their Roman numerals.

ADDITIONAL RELATED PUBLICATIONS

Ridolfo R., **Tavakoli S**., Junnuthula V., Williams D.S., Urtti A., van Hest J.C.M.: Exploring the impact of morphology on the properties of biodegradable nanoparticles and their diffusion in complex biological medium. *Biomacromolecules* 22 (1): 126-133, 2020.

Itkonen J., Annala A., **Tavakoli S.**, Arango-Gonzalez B., Ueffing M., Toropainen E., Ruponen M., Casteleijn M.G., Urtti A.: Characterization, stability, and *in vivo* efficacy studies of recombinant human CNTF and its permeation into the neural retina in *ex vivo* organotypic retinal explant culture models. *Pharmaceutics* 12(7): 611, 2020.

Shakirova J.R., Sadeghi A., Koblova A.A., Chelushkin P.S., Toropainen E., **Tavakoli S.**, Kontturi L.S., Lajunen T., Tunik S.P., Urtti A.: Design and synthesis of lipid-mimetic cationic iridium complexes and their liposomal formulation for *in vitro* and *in vivo* application in luminescent bioimaging. *RSC Advances* 10(24): 14431-14440, 2020.

Subia B., Reinisalo M., Dey N., **Tavakoli S.**, Subrizi A., Ganguli M., Ruponen M.: Nucleic acid delivery to differentiated retinal pigment epithelial cells using cell-penetrating peptide as a carrier. *European Journal of Pharmaceutics and Biopharmaceutics* 140: 91-99, 2019.

AUTHOR CONTRIBUTION

Publication I

The author (S.T) designed the experiments with co-authors. The author conducted most single particle tracking experiments and analyzed their results, wrote the first draft manuscript and revised it with the help of co-authors.

Publication II

The author conducted single particle tracking experiments in the vitreous and analyzed the results. The author participated in the synthesis of HA-DSPE. The author participated in writing the first version of the manuscript and in editing of the manuscript towards final version.

Publication III

The author designed the experiments with co-authors. The author conducted all the experimental work, analyzed the results, wrote the first draft manuscript and revised it with the help of co-authors.

Publication IV

The author designed the experiments with co-authors. The author conducted the in vitro experiments, analyzed the results, wrote the first draft manuscript and revised with help of co-authors.

Unpublished Materials

The author carried out antibody fragment preparation, purification and conjugation to liposomes as well as characterization experiments with help of Dr. Jaakko Itkonen (University of Helsinki) and Dr. Jelle Penders (Imperial College London).

ABBREVIATIONS

AFM	atomic force microscopy
AMD	age-related macular degeneration
CNTF	ciliary neurotrophic factor
DLS	dynamic light scattering
DME	diabetic macular edema
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DPBS	Dulbecco's phosphate buffer saline
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DR	diabetic retinopathy
DSPE	1,2-distearoyl-sn-glycero-3-phosphoethanolamine
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N[amino(polyethylene glycol)]
DSPG	2-distearoyl-sn-glycero-3-phosphoglycerol
Fab'	antigen-binding fragment
Fc	fragment crystallizable
FDA	food and drug administration
FITC	fluorescein isothiocyanate
FTIR	Fourier-transform infrared spectroscopy
GA	geographical athrophy
GCL	ganglion cell layer
GRAVY	grand average of hydropathicity
HA	hyaluronic acid
HC	hard corona
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HSA	human serum albumin
ICG	indocyanine green
IgG	immunoglubin G
ILM	inner limiting membrane
INL	inner nuclear layer

IOP	Intraocular pressure
IPL	inner plexiform layer
IVT	intravitreal injection
Kd	dissociation constant
LAL	limulus amoebocyte lysate
LALS	large-angle light scattering measurements
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LUV	large unilamellar vesicle
Lyso-PC	1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine
MLV	multilamellar vesicle
NFL	nerve fibre layer
NIR	near-infrared
NMR	nuclear magnetic resonance spectroscopy
NP	nanoparticle
nv-AMD	neovascular age-related macular degeneration
ONL	outer nuclear layer
OPL	outer plexiform layer
PBS	phosphate buffer saline
PC	protein corona
PCA	principal component analysis
PDI	polydispersity index
PEG	polyethylene glycol
pI	isoelectric point
PRL	photoreceptors layer
RGC	retinal ganglion cells
RPE	retinal pigment epithelium
SC	soft corona
SEC	size-exclusion chromatography
SPR	surface plasmon resonance
SPT	single particle tracking
SUV	small unilamellar vesicle

TEM	transmission electron microscopy
TKI	tyrosine kinase inhibitor
UPLC	ultra performance liquid chromatography
VEGF	vascular endothelial growth factor
WHO	world health organization

1 INTRODUCTION

Vision is considered as the most important of our senses being vital for independent connections with the world. Given its fundamental role in our life, the loss of vision has a huge negative impact on the quality of life. Currently, 2.2 billion people suffer from some vision impairment; among them tens of millions of patients have severe vision-threatening conditions affecting the back of the eye (World report on vision, WHO, 2019). Aging is the primary factor associated with many of retinal diseases, such as glaucoma, age-related macular degeneration (AMD) and diabetic retinopathy (DR). Considering the current population growth, the number of patients with such diseases will sharply increase in the coming years, particularly in industrialized countries. This situation requires development of effective treatments to many diseases. Currently, intravitreally injected anti-VEGF therapeutics are the most important treatments for AMD and DR [1,2].

Despite significant medical progress during the last two decades, retinal therapy remains challenging because various barriers hinder the delivery of therapeutic agents to the target sites in the retina and choroid [3]. Therefore, topical and systemic routes of administration are not clinically viable options for retinal treatments, since less than 0.001% of applied dose reaches the retina after topical installation of eye drops [4]. For this reason, retina is typically treated using direct intravitreal injections of drugs, for example anti-VEGF biologics, to achieve therapeutic drug levels in the retina and choroid [5]. Injections must be given by specialized nurse or ophthalmologist, often at monthly intervals, because injected drug is rapidly eliminated from the eye. This poses a substantial burden to patients, impose stress on medical personnel, and increase the costs of health care [3]. At the same time, some diseases (e.g. AMD) are not responsive in all patients to the current medications and many retinal diseases are without any drug treatment [6].

Current limitations in retinal drug treatment address the importance to advance ocular drug delivery in order to enable more effective and long-acting treatments. In this regard, nano-sized drug carriers (“nanoparticles”) have been investigated for targeted and sustained drug delivery [7]. They may increase retinal bioavailability, prolong drug retention in the eye, increase patient comfort and minimize adverse drug reactions [7,8]. After intravitreal injection, nanoparticles must transfer from the site of injection to the target tissues, thus highlighting the importance of understanding particle diffusion and interactions with the

vitreous gel [4,9]. Furthermore, retinal access of nanoparticles is restricted by vitreoretinal barrier [10,11], but information on retinal particle penetration is sparse, particularly in relevant animal models.

To bridge this gap, this study was designed to explore the interactions of nanoparticles with ocular barriers, particularly in the case of liposomes, the most commonly used nanoparticles in biomedical applications [12,13]. Important formulation properties, such as surface coating, particle size and surface charge, may alter the pharmacokinetics of intravitreal liposomes, thereby affecting their clinical utility. In this regard, we have systematically investigated the barriers of vitreous humor and vitreoretinal interface using representative animal models. In addition, the potential of liposomal formulations was evaluated in the delivery of sunitinib, small molecule inhibitor of tyrosine kinase, a potential drug for choroidal neovascularization associated with AMD. This thesis provides insights to advance development of nanoparticle-based treatments in ophthalmology.

2 REVIEW OF THE LITERATURE

2.1 Anatomy and Physiology of the Posterior Eye Segment

Human eye is a small yet extremely complex organ, which provides visual perception. The eye can be classified into two segments: anterior and posterior segment (**Fig. 1**). Anterior segment includes cornea, iris, ciliary body, aqueous humor, conjunctiva and lens, but the detailed description of this segment is beyond the scope of this thesis. Posterior segment of the eye refers to the area behind the lens and consists of vitreous humor, retina and choroid.

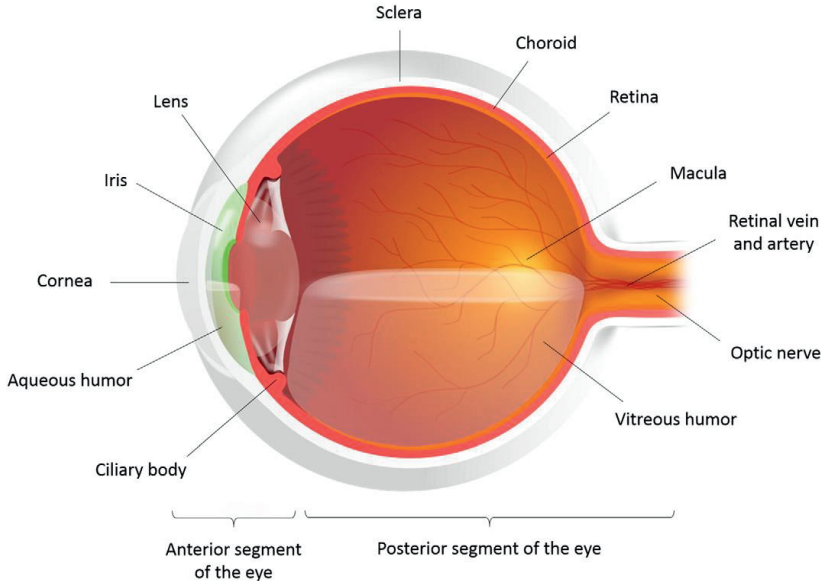

Figure 1 The human eye anatomy. Image reprinted from Delplace *et al., Journal of Controlled Release* 2015, with permission from Elsevier.

Vitreous humor

Vitreous is a transparent gel-like material of approximately 4 ml that occupies two-thirds of the eye volume [14]. Vitreous humor fills the space between the lens and retina and is normally acellular except a few hyalocytes in the cortical vitreous [15,16]. The gel matrix is a highly hydrated (98-99.7% water content) network consisting of structural proteins (collagen type II, IX, V/XI and VI) entangled with highly charged glycosaminoglycans (GAGs) [17]. The main components of vitreal GAGs include hyaluronic acid (HA),

chondroitin sulphate and heparan sulphate. Attraction of water and counter-ions by GAGs provides vitreous with resistance against compressive forces [17,18], while, collagen fibres stabilize the gel state by providing the tensile strength through the intermolecular covalent bonds [19]. Furthermore, vitreous contains several types of non-structural/soluble proteins including albumin, immunoglobulin, transferrin, coagulation proteins and complement factors [20-22]. The protein concentration in healthy human vitreous is between 0.5 mg/ml and 1.5 mg/ml [21-23]. Nonetheless, aging and various pathological conditions can induce changes in the concentrations and biochemical properties of vitreal proteins [24].

Retina

Retina forms the innermost part of solid posterior eye segment tissues. Retina consists of multiple neuronal cell layers (for details, see **Fig. 2**) and the neural retina is considered to be an extension of central nervous system. The neural retina is isolated at the anterior side from the vitreous by inner limiting membrane (ILM). ILM is a basement membrane composed of extracellular matrix (mainly collagen and glycoproteins) and it acts as an anatomical and electrostatic barrier [11,25]. Posteriorly, retinal pigment epithelium (RPE) supports the retina. The outer side of the RPE is lined by acellular Bruch's membrane. The RPE cells form a monolayer with tight junctions and they regulate trans-epithelial transport thereby acting as a blood-retinal barrier (BRB), so called "outer BRB" [4]. RPE restrict the permeability of hydrophilic small molecule drugs as well as macromolecules. Nonetheless, macromolecules such as antibodies (149 kDa) poses 200-300 fold lower inward and outward permeability across the RPE (10^{-8} cm/s) compared to small drug molecules (255-454 Da) [26].

Retinal function is vital for visual perception as it transduces the light information to neural impulses and transmit them to brain via horizontal cells, bipolar cells, amacrine cells, and finally ganglion axons in the optic nerve (**Fig. 2**) [27,28]. Two-thirds of the retina is nourished by the blood supply from retinal arteries, which forms the superficial capillaries near the surface of the retina and send branches to form intermediate and deep retinal capillaries. The outer retina consisting of photoreceptors is avascular in healthy eye, receiving oxygen and nutrients from the choroidal vessels [29]. The endothelium of retinal capillaries form the "inner BRB" which is impermeable to the molecules bigger than 2 nm [4,30].

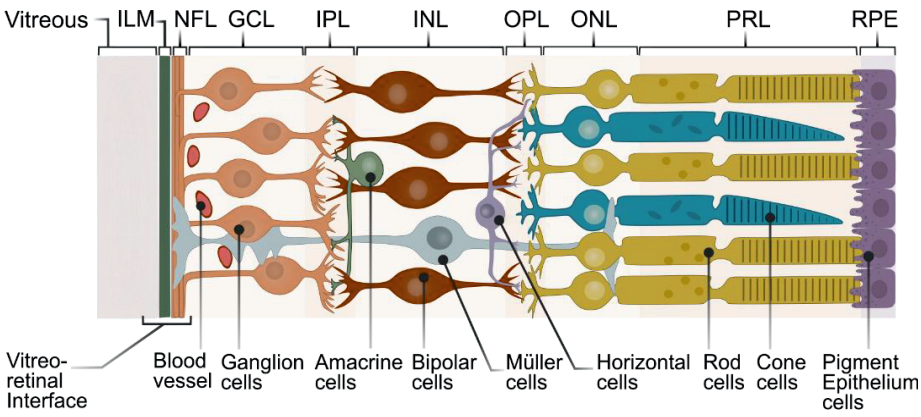

Figure 2 Schematic representation of detailed retinal structure. Retinal layers: inner limiting membrane (ILM), nerve fibre layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptors layer (PRL), retinal pigment epithelium (RPE). Image reprinted from Tavakoli *et al.*, *Journal of Controlled Release* 2020, with permission from Elsevier.

Choroid

Choroid is a thin, densely pigmented and highly vascularized layer located between the retina and sclera. Principal function of the choroid, which accounts for 85% of total ocular blood flow, is to provide the blood supply to the outer retina [31]. Inner part of the choroid is smooth, choriocapillaries, which fenestrate into the Bruch's membrane below the RPE. Unlike the inner part, the the external surface, suprachoroid, is irregular and attached strongly to the sclera [14]. Choriocapillaries are branches of the leaky large choroidal vessels and allows the plasma to diffuse along the Bruch's membrane to nourish the avascular part of the retina especially the photoreceptors. The RPE, however, act as a barrier and prevents the fluid entry to the outer retina except for the nutrients and oxygen [29].

2.2 Posterior Segment Eye Diseases

The main vision-threatening diseases affect retina and choroid. Many diseases of the posterior segment are associated with aging and/or underlying diseases (e.g. diabetes, hypertension, and atherosclerosis) [32,33]. Among these disorders, age-related macular

degeneration (AMD), diabetic retinopathy (DR) and glaucoma are the most prevalent diseases leading to increasing vision loss in aging populations worldwide **(Fig. 3)** [34,35].

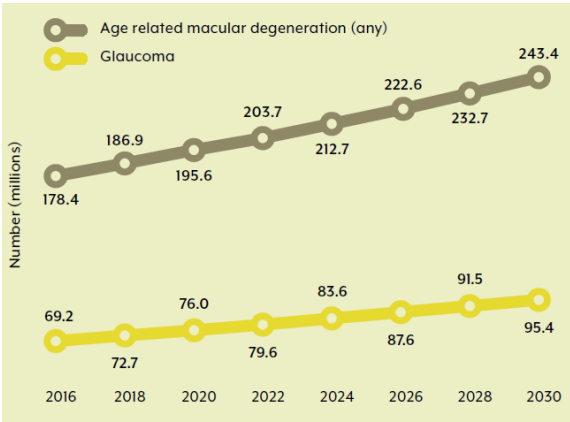

Figure 3. Worldwide projected number of AMD and glaucoma incidence to the year 2030. Adopted from World Health Organization (WHO) report on vision, 2019 [36].

AMD is a progressive breakdown of macula which is a cone-dominated region in the retina [37]. Degenerative process in the macula gradually destroys the visual acuity and central vision. The number of patients suffering from AMD are increasing worldwide and expected to reach over 280 million by the year 2040 (≈1.5 times increase in 20 years) [38]. The complex pathogenesis of AMD is not completely understood. It involves a combination of metabolic, genetic and environmental factors [33,39,40]. The hallmarks of AMD include intracellular protein aggregates in the RPE and extracellular "drusen" deposits of lipids, proteins and complement factors in Bruch's membrane [41]. Formation of drusen gradually causes perturbed exchange of oxygen and metabolites between choriocapillaries and RPE [42].

Clinically, the AMD is classified to dry-AMD and neovascular-AMD (also known as wet-AMD) [39]. Dry-AMD is a more common type of AMD and accounts for 85-90% of diagnosed cases [43]. In the early stages, dry-AMD is defined by small to intermediate-sized drusens, without significant sign of vision loss. In advanced stages, so called "geographical atrophy (GA)", large drusen prevents vascular supply from choriocapillaris, resulting in malfunction, cell death in RPE and photoreceptors, eventually leading to loss of vision [37]. The neovascular form of AMD (nvAMD) involves pathological sprouting of new abnormal blood vessels to the outer retina and subretinal space [29]. Origin of these

vessels may be in the deep retinal capillary bed or choroidal vessels (choroidal neovascularization, CNV) [29]. Neovascularization may lead to accumulation of fluid and blood due the leakiness of neo-vessels. Pathogenesis of nvAMD is associated with an increased production of angiogenic growth factors, such as vascular endothelial growth factor (VEGF) [29,44]. However, evidences indicate also a link between immune-mediated events and neovascularization, suggesting that the elevated VEGF alone does not lead to nvAMD [33,45]. Among all AMD cases, 10-20% develop the neovascular form that causes much faster loss of vision than dry-AMD [37,46].

Diabetic retinopathy (DR) is the most common vision-threatening complication of diabetes [47]. Chronic hyperglycemia causes functional and structural damage to retinal capillaries and BRB breakdown. Increased permeability of retinal vessels results in blood and fluid leakage to the back of the eye (microaneurysms, retinal hemorrhages) [47,48]. Accumulation of fluid in macula induces diabetic macular edema (DME) with inflammation and swelling of the macula, but this is less prevalent than DR [48,49]. In the advanced stage of DR, also known as proliferative DR, hyperglycemia can lead to retinal microvascular closure and retinal ischemia. As a result, hypoxic condition mediates the over-expression of VEGF that stimulates retinal neovascularization [29,50]. Retinal neovascularization originates from retinal vessels and often penetrating in the ILM and growing into the vitreous [50].

Glaucoma is a neurodegenerative disorder often caused by age-related increase of intraocular pressure (IOP) that may damage the inner layer of the retina (retinal ganglion cells, RGCs), and the optic nerve [39,51,52]. Elevation in IOP is usually associated with perturbation in aqueous humor outflow from trabecular meshwork in the anterior part of the eye [53]. However, decreased age-related flexibility of the sclera can also induce the increased IOP [54]. Glaucoma is an important cause of irreversible vision loss **(Fig. 3)** [2].

2.3 Current Drug Delivery Strategies to the Posterior Segment of the Eye

The primary goals of any ocular drug delivery system are to maintain therapeutic drug concentration at the target site at long enough dosing intervals. Since drug concentrations at target sites depend on drug penetration across the barriers, it is essential to understand ocular barriers in drug development.

Ocular barriers are classified as static anatomical and dynamic physiological barriers that are essential in protecting the eye from xenobiotics, yet they pose challenges in ocular drug delivery [55]. The impact of barriers on drug delivery depends on the route of drug administration (**Fig. 4**).

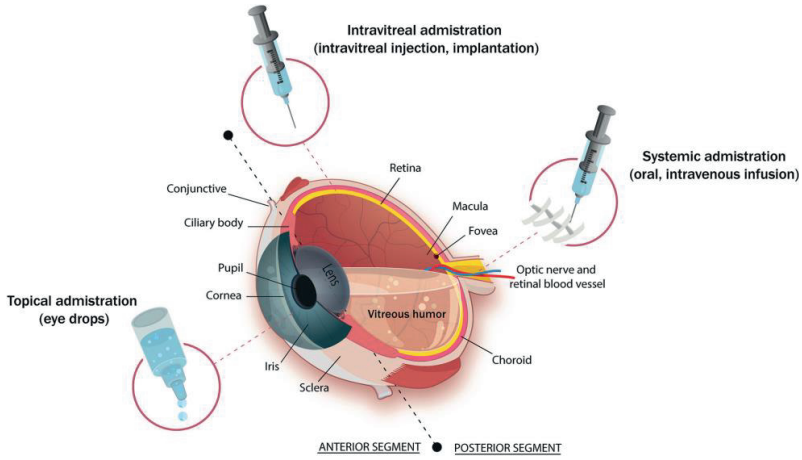

Figure 4. Commonly used ocular route of administration. Image reprinted from Ilochonwu *et al. Journal of Controlled Release* 2020, with permission from Elsevier.

Topical instillation is the most common method of ocular drug administration. It is non-invasive and applicable in the home treatment of out-patients. In clinical practice, topical ocular formulations (eye drops, ointments, gels) are used to treat anterior segment diseases, such as dry eye, cataract, allergic conjunctivitis, infections and reduction of eye pressure in glaucoma [56,57].

Poor drug bioavailability after topical administration does not lead to therapeutic drug concentrations in the posterior segment. Following topical administration, only 0.1 - 7 % of small molecular drugs reach the aqueous humor [58,59]. Such low absorption is due to the rapid pre-corneal drug loss by drainage of eye drop, tear turnover (1 µl/min) and systemic absorption across conjunctiva [3,60,61]. Besides pre-corneal loss, the multi-layered cornea poses an anatomical barrier that limits ocular drug absorption [62]. The cornea is composed of three main layers (epithelium, stroma, endothelium) of which the anterior tight epithelium significantly limits drug absorption, particularly the large and hydrophilic drug molecules [63-65]. Permeation of lipophilic small molecule drugs takes place mainly via transcellular route [66]. In addition to the trans-corneal route, topical drug may be absorbed through conjunctiva and sclera, across non-corneal route [67]. This route is

relevant in absorption of large and hydrophilic drugs [68], because conjunctival epithelium is leakier than the corneal epithelium [69]. Nonetheless, significant fraction of instilled drug dose (34-79%) is systemically absorbed into the blood circulation across conjunctival sac [68,69] and only the portion that is not eliminated by blood circulation reaches the sclera and may partly gain access to the choroid and retina. Altogether, even in the best cases, less than 0.001% of the topical dose reaches the retina, resulting in therapeutically inadequate drug concentrations [70,71].

Systemic route, including parenteral and per oral administration, can deliver drugs to the retina and vitreous through ocular blood flow. However, the process is hindered by BRB tight junctions in retinal capillaries' endothelium (inner BRB) and RPE (outer-BRB). In the similar manner, blood-aqueous barrier (BAB) in iris capillaries and ciliary endothelium prevent the drug entry into the posterior segment from blood stream. Moreover, efflux transporters in the RPE cells may limit access of drugs from blood stream to the retinal targets [72]. Other limiting factors include drug dilution in blood circulation, plasma protein binding and systemic clearance that significantly restrict retinal delivery of systemic drugs [73]. Consequently, this route may only be useful for small lipophilic drugs with broad therapeutic window (such as antibiotics) that can be administered in high and frequent doses to treat posterior segment diseases [3,74].

Intravitreal (IVT) injection is the current gold standard in drug administration to the posterior segment of the eye. IVT injection has been investigated for various pharmaceutical preparations, such as solutions, suspensions, micro/nano-particles and implants [75]. Direct delivery of therapeutics into the vitreous, provides immediate intraocular drug delivery and minimizes the required drug dose and systemic side effects. Although this route bypasses many barriers, there are still several barriers that must be taken into account in drug development [76].

Vitreous itself is the first barrier that must be overcome after IVT injection. After intravitreal administration, drug distribution depends on the compound properties (e.g. size, charge), and state of the vitreous [4]. The gel-like matrix of vitreous limits diffusion of large particles (> 550 nm), and particularly positively charged particles due to the electrostatic interactions with negatively charged hyaluronic acid [17,77,78]. In contrast, small drug molecules or protein drugs are almost freely mobile in the vitreous [4]. By aging, vitreous undergoes progressive liquefaction (synchysis) and collagen fibre

aggregation (syneresis) causing partial loss of gel-state and reducing the barrier role of the vitreous [18,79].

Physiological factors, such as intraocular convection and clearance pathways, can also affect drug distribution and elimination in the vitreous. Convection in posterior direction does not influence the distribution of small molecules, but it might affect distribution of larger compounds or particles [76,78]. Vitreal drug clearance takes place via two main routes: 1) anterior route to the anterior chamber and elimination via aqueous humor turnover; 2) posterior elimination across the BRB [31]. The elimination rate and route of intravitreal therapeutics depends on their physicochemical properties. Large hydrophilic compounds (e.g. proteins) and particulate systems do not penetrate the BRB, and are mainly eliminated via anterior route, resulting in half-lives of several days [3,4]. Small drugs, particularly lipophilic compounds, are cleared via posterior route leading to the short intravitreal half-lives (<10 h) [80,81]. Therefore, their IVT administration as simple solutions, without sustained drug release, is not practical [80]. Since ocular half-life of small molecule drugs (<1000 Da) in general is less than 1 day, chronically used IVT injections are macromolecules (>50 kDa), such as potent anti-VEGF agents, with half-lives in the range of several days [31]. Even though concentration of endogenous vitreal proteins is much lower than in the plasma, protein binding may alter the drug levels in the vitreous, prolonging vitreal half-lives [21,24]. Nonetheless, a recent study on vitreal binding of 35 small molecule drugs suggests that protein binding may only modestly affect the drug half-life in the vitreous [24], while the half-life of 40 kDa nanobody was increased by 3-fold with a high affinity binding to albumin [24,82].

Retinal penetration is essential to obtain the therapeutic efficacy after IVT injections. In this respect, therapeutics must overcome vitreoretinal interface and inner limiting membrane (ILM), which is a basement membrane separating the vitreous from retina [83]. ILM is mainly composed of collagen type IV, laminin and negatively charged proteoglycans that form a physical barrier for retinal delivery [84,85]. Retinal permeation across the ILM depends on multiple factors, such as compound or particle properties (e.g. size and charge) and endogenous factors (e.g. ILM thickness, aging, disease-related changes, morphological differences) [84]. Moreover, the ILM properties differ between species [11] and the ILM thickness and composition may become stiffer by aging [86]. For example, at older age the concentration of collagen type IV may increase, while levels of

laminin may decrease [86]. The thickness of foetal ILM is about 70 nm and later it will become thicker, reaching 2 μm (TEM) or 4 μm in the posterior pole (based on atomic force microscopy (AFM) measurements) [86-88]. In the fovea and at the rim of optic nerve, the ILM is rather thin (< 140 nm), which may be essential for the normal vision [89-91]. ILM thickening can be associated with the slow degeneration of the collagen fibres, while the protein synthesis goes on at the vitreoretinal interface during the entire life-span [86,92]. Besides age-related changes, the properties of ILM might be altered in disease state. Diabetes-related ILM thickening and increased collagen type IV synthesis have been reported in long-term diabetes [93]. ILM might be even broken in proliferative diabetic retinopathy [94,95].

Negatively charged components of the ILM restrict the permeation of cationic compounds, while the anionic and neutral drug molecules or drug delivery systems are less hindered by this barrier, unless their size becomes a limiting factor [11,76,96]. According to Pitkänen *et al.*, retinal permeation of intravitreal macromolecules and particles is predominantly influenced by the charge of the permeant. It was evident that FITC-dextran of 2000 kDa (mean molecular weight) and negative charges diffused into the retinal layers, but 20 kDa positively charge FITC-poly-L-lysin failed to pass across bovine ILM [97]. In addition, several studies suggest that the retinal permeation of the macromolecules depends on the molecular weight [85,98-100]. According to these investigations, Fab' fragments (48 kDa) diffuse into the retina, while there is a controversy on the retinal permeation of the full-length antibody such as bevacizumab (148 kDa) [101]. Transient enrichment of the antibody at the ILM prior to retinal permeation was evident in many of observations [11], but the extent of retinal permeation of full-length antibodies remains unclear.

Other local routes of administration: Drug delivery to the posterior segment can be accomplished via other route of administrations such as subretinal, periocular and suprachoroidal [102,103]. Subretinal route bypasses the ILM barrier, because drug is injected directly between the RPE and photoreceptors. However, these injections require substantial expertise and repeated injections are not feasible [93]. In contrast, periocular drug administration is less complicated, involving injection of drug solution or suspension into the subtenon or subconjunctival space. Such injections are widely used in anterior segment drug delivery and they are less invasive than IVT injections. However, the barriers (sclera, RPE, conjunctival and chroroidal blood flows) limit retinal bioavailability

to about 0.1% [104-106]. Subtenon injection is more effective than subconjunctival injection, resulting in 5-fold increase in bioavailability, but still the levels are low [107]. In suprachoroidal injection, the drug is delivered to the space between the sclera and choroid. The sclera is bypassed with this method offering higher bioavailability compared to periocular route [108]. In this case, retinal bioavailability is limited by choroidal blood flow and the RPE, but choroidal bioavailability is nearly complete. However, choroidal blood flow removes drug rapidly after injection unless special formulations are used. Suprachoroidal delivery is still at experimental stage, not yet in the clinical practise (e.g. suprachoroidal microneedles, phase III of clinical trial) [109,110].

2.4 Current Therapies for the Posterior Segment of the Eye

Increasing prevalence of posterior segment eye diseases in aging population demands development of effective therapeutics. In this respect, many experimental and clinical ocular drug products have been designed for different routes of administration and duration of action. Inflammation and elevated levels of VEGF are recognized as major features in many retinal and choroidal diseases such as AMD, CNV, DR, DME and retinal vein occlusion [111,112]. Therefore, the most important and promising medications include anti-VEGF agents, anti-inflammatory drugs and neurotrophic factors that are given as IVT injections and implants [4]. Also, systemic administration of liposomal verteporfin as photodynamic therapy is still in clinical use. In this case, verteporfin (approved in 2000) produces short-lived oxygen free radical in the presence of laser light to destroy blood vessels [113]. It is indeed the only systemic treatment for nvAMD, but its efficacy does not match that of IVT anti-VEGF therapy. Photodynamic therapy requires frequent visits to the clinics that leads to poor patient compliance.

2.4.1 Intravitreal Anti-VEGF

IVT injection of anti-VEGF agents is the most promising strategy for posterior segment of the eye diseases [5]. Blocking the VEGF-pathway can inhibit the pathological vessel growth and leakiness. In this respect, the most common strategy is to prevent binding of VEGF-A to its receptors. The first VEGF-specific humanized monoclonal antibody, bevacizumab (Avastin®), was approved by the U.S. Food and Drug Administration (FDA) in 2004 for metastatic colorectal cancer [5]. Bevacizumab binds to all isoforms of VEGF-A

and VEGF-B and it is widely used off-label in nvAMD and DME. Investigations on VEGF-mediated ocular neovascularization led to development of pegaptanib (Macugen®, PEGylated aptamer against $VEGFA_{165}$) and ranibizumab (Lucentis®, Fab' fragment of bevacizumab) which received FDA approval for nvAMD in 2004 and 2006, respectively [5,112]. Soluble VEGF receptor aflibercept (Eylea®) was approved in 2011 for IVT injection in nvAMD and all stages of DR. It is a recombinant fusion protein, also known as VEGF Trap, consisting of binding domain of VEGF receptor-1 (VEGFR-1) and VEGFR-2 fused to Fc fragment of human IgG1 [114]. Aflibercept binds to all isoforms of VEGF-A and VEGF-B at higher affinity than bevacizumab [5].

All currently approved anti-VEGF biologics are formulated as sterile solution in single-dose vial or pre-filled syringes for IVT injection (maximum volume of injection is 100 µl). Given the hydrophilicity and molecular weight of these macromolecules, approximately 90% of the dose is eliminated through anterior route resulting in vitreal half-lives in the range of a week [57]. Hence, injections at 4 to 8 weeks intervals are needed for anti-VEGF proteins [112]. The IVT injection interval has been extended to 8-12 weeks in the most recently approved (2019) anti-VEGF agent brolucizumab (Beovu®) that is a humanized monoclonal single-chain Fv (scFv) antibody fragment [115]. It binds to major isoforms of VEGF-A, including $VEGFA_{165}$ [116]. Also, abicipar pegol, antibody mimetic small protein against VEGF-A, has potential to stabilize vision at 12-weeks dosing intervals based on the Phase III clinical trial results, but FDA did not approve it for nvAMD treatment due to the incidence of intraocular inflammations in mid-2020 [117]. Despite the substantial benefits of such therapeutic options, there are unresolved challenges in the treatment of posterior segment diseases. For instance, one-third of the patients with DR are not responsive to anti-VEGF treatment [118]. Therefore, photodynamic therapy remains the only treatment option in those cases until more effective therapy to target leaky retinal blood vessels will be introduced [119]. Likewise, almost 40% of nvAMD patients demonstrate sub-optimal response to the anti-VEGF treatment [120]. Current approved doses are maximal and no extra efficacy can be achieved at higher doses in nvAMD and DR [121]. Currently, clinical under-treatment is partly related to inadequate number of injections that is due to the reduced patient compliance. The IVT injections are occasionally associated with rare, but serious, adverse effects, such as retinal detachment, increased IOP, retinal haemorrhage, cataract and endophthalmitis [122-124]. In addition, IVT injections must be performed by

ophthalmologists or expert nurses and they impose a major burden on healthcare. Prolonged duration of the injections would be beneficial.

2.4.2 Corticosteroids

Considering the substantial evidence on the underlying role of inflammation in the pathogenesis and progression of retinal diseases, one treatment strategy is to block the inflammatory pathways. Intravitreal corticosteroids, such as dexamethasone and fluocinolone acetonide, have shown anti-inflammatory and anti-angiogenic properties resulting in promising outcomes in DME, particularly in its advanced stages [125]. The half-lives of injected small molecule solutions are only a few hours, since they permeate through BRB posteriorly [4,57]. Suspension dosage form of small molecule drugs including corticosteroids, however, can prolong the vitreal retention owing to the slow dissolution rate [126]. For instance, IVT suspensions of triamcinolone acetonide (Triesence® and Trivaris®) showed the extended vitreal residence time of up to a few months [127]. IVT corticosteroids are also formulated as intravitreally injectable implants that are used at 6-month (dexamethasone, Ozurdex®) or 36-month (fluocinolone acetonide, Iluvien®) intervals [128]. The implants avoid the side effects of multiple IVT injections, but the vitreous traction and long-term corticosteroid therapy may be associated with cataract and/or elevated IOP [129,130]. Furthermore, over longer time periods, they may increase the risk of glaucoma and systemic side effects, such as gastrointestinal upset, hypertension and osteoporosis [131,132].

2.5 Emerging Therapies and Drug Delivery Systems

Over the past two decades, several therapeutic agents have been approved for nvAMD, DME and DR, and many more are in the clinical trials. Consequently, continuous efforts have focused on 1) targeting multiple pathways that are linked with pathological neovascularization and 2) developing the innovative drug delivery systems for existing drugs to attain prolonged therapeutic concentration at the target site thus avoiding repeated IVT injections [133].

In this respect, other VEGF signalling pathways have been explored. One strategy is to block the VEGFR signalling by tyrosine kinase inhibitor (TKI) drugs (e.g. sunitinib,

axitinib, vorolanib, pazopanib) in order to stop the neovascularization [5,134,135]. GB-102 (GrayBug Vision™), reservoir of sunitinib maleate in polymeric microparticles, is a potential sustained-release IVT formulation for the treatment of nvAMD that is in phase II clinical trial [136]. In addition, sunitinib has shown neuroprotective effect by blocking the dual-leucine zipper kinase (DLK inhibitor) which makes it an interesting option for treatment of retinal disorders [137,138]. IVT axitinib implant (OXT-TKI, Ocular Therapeutix™) has reached phase I clinical trial for the treatment of nvAMD and DME [135,139]. Similarly, Durasert™-TKI (EyePoint™) implant has been investigated for vorolanib delivery in preclinical studies for nvAMD and DR treatment [140,141]. In another study, per oral pazopanib was used in CNV mouse model to suppress neovascularization via inhibition of VEGFR and platelet-derived growth factor (PDGF) receptor [142].

Blocking the VEGFR-2, the main mediator of neovascularization, is another intriguing strategy. Anti-VEGFR-2 monoclonal antibodies such as tanibirumab and ramucirumab (Cyramza®) suppress the neovascularization by inhibiting the endothelial cell migration and proliferation [5,143]. This effect was observed in preclinical studies on laser-induced CNV rat model, but there are no ongoing clinical studies based on this approach.

Given the multiple mechanisms in retinal and choroidal disorders, more efficient outcomes may be attained by targeting multiple pathways of neovascularization. Preclinical studies on PDGF inhibition in combination with anti-VEGF-A agents showed promising results for the treatment of nvAMD [144]. This approach is in phase III trials using a combination of pegpleranib (Fovista®, anti-PDGF aptamer) and ranibizumab [140]. In addition, faricimab is under investigation in phase III clinical trial for the treatment of DME and nvAMD. Faricimab is a bispecific antibody targeting angiopoietin-2 and VEGF-A signalling pathways to stabilize the blood vessels and limit permeability, which has shown enhanced efficacy over anti-VEGF monotherapy [6].

Some posterior segment eye diseases are characterized by inflammation (immune cell infiltration) and neural cell degeneration, but anti-VEGF compounds do not affect these factors [145]. In addition to corticosteroids, inhibition of complement proteins (e.g. C3, C5 and C9) have shown potential in AMD treatment [146-148]. Hemera Biosciences developed a viral-vector mediated gene therapy (HMR59) to inhibit the C9 complement cascade. This product is in phase I clinical trial for nvAMD [146].

Various cytokines and growth factors protect neural retina from degeneration. For instance, ciliary neurotrophic factor (CNTF) showed preclinical protective properties against neurodegenerative disorders (e.g. in glaucoma). However, CNTF has a short vitreal half-life necessitating frequent IVT injections [129]. Therefore, IVT implant (Renexus®) was developed utilizing encapsulated cell technology (ECT) in which genetically-modified cells secrete CNTF to the vitreous over a prolonged time [149]. This approach is in Phase III of clinical trial for glaucoma [150,151].

During the past two decades, gene delivery gained interest for treatment of posterior segment eye diseases such as AMD, glaucoma and some inherited retinal diseases [152-155]. Herein, therapeutics including DNA, mRNA and regulatory RNAs (e.g. siRNA and miRNA) must be shuttled into their specific cytosolic or nuclear targets in retinal cells such as RPE and photoreceptors [156,157]. The route of administration depends on the target site, yet, given their negative charge, large molecular weight and the lability of these compounds in biological environment, carrier systems (viral and non-viral) are usually required for intracellular delivery. Despite several advantages of non-viral carriers, such as lower immunogenicity and higher loading capacity, viral-based carriers have demonstrated the most effective transfection of retinal cells [158]. Viral vectors are mainly based on modified adeno-associated virus (AAV) family [140,159]. Most successful retinal nucleic acid transfer experiments have relied on sub-retinal injections, because vitreoretinal interface hinders the access of the viral and non-viral particles to the retina. Strategies to overcome this barrier are thus needed. Recently, clinical trial on intravitreal injection of ADVM-022 was launched, involving AAV vector carrying cDNA for aflibercept, (phase I clinical trial for nvAMD) [160]. This approach offers durable expression of anti-VEGF proteins following a single dose administration for treatment of nvAMD.

Parallel to the emerging therapeutics, innovative drug delivery systems and strategies have been developed to prolong the dosing interval of anti-VEGF therapeutics and corticosteroids. Table 1 shows examples of systems for posterior segment eye diseases.

Table 1. Long-acting delivery systems for the treatment of posterior eye segment

Drug	Delivery system and Route of administration	Indication	Dosing interval	Status	Ref.
Ranibizumab	Port Delivery System (PDS), surgical implantation across the sclera into vitreous	nvAMD, DME and DR	6-12 months interval between refills	Phase III clinical trial Phase III trial for nvAMD has been completed	[161, 162]
Ranibizumab	Posterior Micro Pump (PMP), surgical implantation into episclera	DME	3 months	Studied in prospective small-scale clinical trial	[163, 164]
Aflibercept (OXT-IVT)	Shape-changing implant, IVT	nvAMD	4-6 months	Preclinical studies	[139]
Aflibercept (OXT-AFS)	Thermosensitive hydrogel depot, suprachoroidal	nvAMD and DME	6 months	Phase I clinical trial	[139]
Triamcinolone acetonide (Xipere®)	Microneedles, suprachoroidal	DME	3 months	Phase III clinical trial	[55, 126]

Nevertheless, most aforementioned technologies require invasive administration methods, such as surgical procedures to implant or to remove devices. Consequently, nanotechnology-based drug delivery systems may offer a promising alternatives to overcome some of the limitation of current therapies, particularly by providing possibilities for retinal permeation and cellular delivery.

2.6 Nanotechnology-based Drug Delivery Systems

Nanotechnology has gained significant research interest in medicine during the past decades. In this regard, the use of nano-sized carriers (below 1000 nm in diameter) have been investigated for drug delivery to ocular target tissues in order to manage the posterior segment disorders. Nanoparticles may enable increased intraocular retention, extended drug release and distribution to the tissues. As a result, they may improve the efficacy of treatment efficacy, enable the use of difficult compounds as therapeutics and prolonging the drug dosing intervals [118,129].

Nanoparticles can be classified based on their composition. Numerous types of materials have been applied for ocular drug delivery systems, such as synthetic polymers (e.g. polymersomes, polymeric micelles, and hydrogels), proteins (albumin nanoparticles), lipids (e.g. liposomes, solid lipid nanoparticles (SLN)), and inorganic compounds (e.g. gold-nanoparticles) [118,165]. To this end, intravitreal injection of nanoparticles have been explored for retinal delivery of various therapeutic compounds, such as small molecule drugs, peptides, proteins and small regulatory RNAs [7,8,166,167].

2.6.1 Nanostructured drug delivery systems for retinal and choroidal diseases

Polymeric nanoparticles have been studied as sustained drug delivery systems for back of the eye disorders. The most commonly investigated polymers include PLGA copolymers (poly (lactide-co-glycolide), PLA (poly lactides), PCL (poly (caprolactone)), poly (methyl methacrylate), chitosan and hyaluronic acid (HA) [7,163]. FDA approved PLGA gained interest in ocular drug delivery based on its biodegradability and biocompatibility [7,118]. Several preclinical IVT studies have utilized PLGA in polymeric nanoparticles to control the choroidal neovascularization and retinal degeneration. Prolonged inhibition of neovascularization over 6 weeks has been observed with sustained release bevacizumab-loaded PEG and PLGA nanoparticles (particle size = 819 nm) and dexamethasone-loaded PLGA nanoparticles (particle size = 253 nm) in laser-induced CNV rat models [168,169]. Recently, polymersomes (particle size = 100 nm) and polymeric micelles showed enhanced vitreal half-life of 32 days and 9 days, respectively, in rabbits suggesting a promising retinal drug delivery system (unpublished).

Besides synthetic polymers, endogenous protein, such as human serum albumin (HSA), has been evaluated for retinal drug delivery via IVT administration. The albumin

nanoparticles were first approved by FDA for intravenous delivery of paclitaxel (Abraxan®) in breast cancer treatment, but given its numerous interesting properties for extended drug delivery, it also gained interest for ophthalmic application [170]. In this respect, Kim *et al.* demonstrated that HSA nanoparticles (particle size = 152.8 nm) loaded with small drug molecule (brimonidine) have neuroprotective effect in optic nerve crush rat model lasting up to 14 days [171]. *In-situ* forming hydrogels are another polymeric-based delivery systems which have recently received attention for long-term release of biologics following the IVT administration [172,173]. For instance, hyaluronic acid (HA)-dextran hydrogels showed sustained delivery of bevacizumab at the therapeutics concentration for up to 6 months in rabbits [174].

Solid-lipid nanoparticles (SLNs) offer several beneficial features, such as controlled release of hydrophobic and hydrophilic drug molecules, biocompatibility, stability and ease of production. Nonetheless, the limited loading capacity of SLNs restrains its application for prolonged ocular drug delivery [175]. Instead, SLNs were successfully used as gene vectors for plasmid transfection of photoreceptors in mouse models, preventing loss of photoreceptors 2 weeks after IVT injection [176]. Nano-structured lipid carriers (NLCs) are more advanced generation of lipid-based nanoparticles with higher drug loading capacity compared to SLNs [7]. In the eye, NLCs have been investigated mostly for the anterior segment drug delivery. Araujo *et al.* explored the use of NLCs for triamcinolone acetonide delivery to the posterior segment via topical administration in mice, yet, no drug was detected in the intraocular tissues after 160 min [177].

2.6.1.1 Stimuli-responsive nanoparticles

Besides sustained-release capability, nanoparticles can be designed in order to provide stimuli-responsive drug release. In this respect, the external signals, such as light, heat, and pH are used for triggered drug release [166,178]. Given the unique structure of the eye, light-triggered drug release is an intriguing solution that can be leveraged for extending the IVT injection intervals of both lipid-based and polymer-based nanocarriers [179]. Light-triggered release of nintedanib-loaded polymeric nanoparticles is an example of such design, which allowed 10 weeks of CNV suppression in rat model [180]. Moreover, light-triggered hydrogel systems based on agarose-coated gold- nanoparticles showed triggerable release of bevacizumab for 6 months [181].

2.6.1.2 Target-specific nanoparticles

Another valuable feature of the nanoparticles is their potential for target-specific delivery to retinal cells via surface engineering. Particularly, polymeric materials can be modified with ligand conjugation towards fabrication of targeted drug delivery systems. Ligand-targeted drug delivery, so called “active targeting”, is based on specific interactions between a ligand and receptor in the target cells (e.g. folate-receptor mediated targeting to the RPE cells) [166]. Delivery of triamcinolone acetonide with folate-coated PEG-PCL nanoparticles into ARPE-19 cells was significantly higher than with uncoated carriers [182]. Hyaluronic acid (HA) is also a potential targeting moiety that has been applied for drug delivery to CD44-expressing cells, such as Müller cells and RPE cells [183-185]. For instance, HA-modified HSA (particle size = 252.7 nm) could significantly improve therapeutic efficiency of connexin-43 mimetic peptide (Cx43 MP) to suppress the inflammatory process and prevent the RGCs loss in rats with retinal-ischemic condition [186]. Furthermore, VEGF-grafted magnetic nanoparticles (nanomag®) revealed promising retinal drug delivery strategy through cell-specific targeting in zebrafish [187].

2.7 Liposomes for the Posterior Segment Eye Diseases

Liposomes were first introduced as drug delivery systems for cytotoxic agents in 1965 and nowadays several clinically approved liposomal formulations are used, including Doxil®, Ambisome®, DaunoXome®, DepoCyt®, Mayocet®, Visudyne®, Lipo-Dox® DepoDur™, Marqibo®, Onivyde™ and Vyxeos® [176,188]. Liposomes are spherical vesicles that include an aqueous core encapsulated with lipid bilayers of amphiphilic phospholipids (e.g. phosphatidylcholine and phosphatidylethanolamine) [189]. Given their biphasic structure, hydrophilic and hydrophobic drug molecules can be entrapped in the aqueous compartment and lipid bilayer, respectively. Liposomes are classified as SUV (small unilamellar vesicles, 20-200 nm), LUV (large unilamellar vesicles, 200-1000 nm) and MLV (multilamellar vesicles, > 500 nm) [190]. Intravitreal liposomes are typically in the submicron size range [191]. In general, lipid film hydration results in MLVs, which can be further processed to form SUVs via sequential extrusion through polycarbonate filter membranes to achieve desired particles size. Sonication and microfluidics are alternative methods for particle size adjustment.

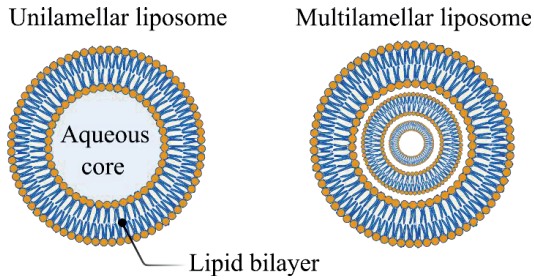

Figure 5: Schematic representation of different liposome structures.

Liposomes offer several advantages as an ocular drug delivery system including high biocompatibility, stability, robustness and ease of production, high loading capacity and biodegradation [192]. Furthermore, they can prolong the intraocular half-life [126]. In this respect, the lipid-bilayer permeability can be tuned with the choice of lipid composition; saturated phospholipids with long hydrocarbon chains provide more stable and less leaky bilayers [189]. From the pharmaceutical point of view, liposomes can improve the solubility of poorly water-soluble drugs, such as corticosteroids, TKIs and IOP-lowering agents [166]. Moreover, they can protect the macromolecules (e.g. proteins and RNAs) from enzymatic degradation. The net charge of the liposomes affect their ability to overcome ocular barriers. Liposomes are commonly composed neutral, cationic and/or anionic lipids and, in addition, PEG-conjugated lipids are used to prepare "stealth-liposomes" with improved stability and intravitreal half-life [192]. In addition, surface-coating with HA, an endogenous vitreal component, is an interesting alternative to PEGylation [183,193].

To date, various intravitreal liposomal formulations have been investigated for the ocular sustained drug delivery. Tacrolimus-loaded liposomes, for instance, resulted in significant reduction of intraocular inflammation in retinitis rat model over 14 days following the IVT injection [194]. In addition, liposomes were successful in many preclinical studies to prolong the therapeutic concentrations of antibiotics (e.g. amphotericin B, ciprofloxacin) [195,196]. This indicates the potential of liposomal carriers to increase the efficacy of hydrophobic small drug molecules. In addition, IVT injection of liposomal bevacizumab showed five-times higher vitreal drug concentration compared to bevacizumab alone after 42 days [197]. Beside drug delivery, liposomes have been explored for gene delivery to retinal targets. In gene therapy by liposomes, the main lipid components are cationic for

binding and condensation of the nucleic acids [198]. The formulation are often PEGylated to provide stability in the vitreous [198]. Intravitreal administration of PEGylated liposome-protamine-HA loaded with siRNA (particle size = 132 nm) resulted in reduction of neovascularization at least for 2 weeks by inhibition the VEGFR-1 expression in laser-induced CNV rat model [199].

Targeted drug delivery to the posterior segment of the eye is feasible via either passive or active targeting [7]. In the passive mechanism, liposomes diffuse across permeable membranes without any ligands, while active targeting involves functionalization of the liposome surface with targeting ligands that will bind to ocular target cells [12,200]. In this respect, HA-coated lipoplexes showed eight-fold higher transfection efficiency compared to unmodified liposomes in the ARPE-19 cells [193]. In addition, Wang *et al*. designed a targeted liposome via peptide-conjugation, 12-aminoacids peptide coded as YSA, in order to reach specific delivery of doxorubicin to the RPE cells. The *in vivo* study of YSA-liposomes in CNV rat models showed significantly higher inhibition of neovascularization compared to unmodified liposomes [201]

As it was discussed in the previous section, the concept of activated drug release is of great interest in the field of ocular drug delivery. In particular, the light-responsive formulation would be attractive as the eye is accessible to light. This strategy was studied in several clinical trials for photodynamic intravenous therapy of nvAMD using Visudyne® (liposomal verteporfin) and IVT anti-VEGF antibodies such as ranibizumab and bevacizumab to manage the CNV [179,202]. Later, Lajunen *et al.* developed new type of light-activated liposomes with on-off light triggered release mechanism [203]. Toward this goal, the photosensitizer (indocyanine green (ICG)) is loaded to the liposomal membrane for temporal and spatial controlled drug release. Herein, ICG converts the near-infrared (NIR) laser energy to heat, consequently converting the membrane leaky, thereby allowing drug release [203,204].

Altogether, liposomes can be considered as potential drug delivery system to transport therapeutic compound into the retina to obtain more efficient and long-acting treatment.

2.8 Models to Study the Retinal Drug Delivery

Despite some successful preclinical studies on nanoparticle-based retinal drug delivery, their translation to clinical use remains challenging owing to complicated ocular barriers that limit access to the posterior segment tissues. Several barriers are avoided by IVT injection, yet for successful retinal drug delivery the nanoparticles must overcome two main hurdles after IVT administration: vitreous and ILM. Consequently, it is essential to explore whether the nanoparticles are able to overcome these barriers to reach retinal targets. Currently, most preclinical results on retinal nanoparticle delivery are based on *in vivo* studies with mice and rats. Given the small vitreous volume and structurally different ocular barriers, translation of such observations from the rodents to clinical application is troublesome. Over the last two decades, several *in vitro* and *in vivo* methods have been investigated to expand our understanding on interactions of drug delivery systems with the ocular tissue barriers.

2.8.1 Methods to study the barrier role of vitreous

The vitreous may restrict diffusion of nanoparticles as discussed in section 2.3. The mobility in the vitreous depends on the characteristics of the particles as well as the physiological state of the vitreous. Particle size, surface charge, surface coating (e.g. PEG and hyaluronic acid), material properties and vitreal-nanoparticle interactions (protein binding and interaction with other components of the vitreous) can influence the diffusion of nanoparticles in the vitreous. In the early *in vitro* studies, diffusivity of particles was measured in isolated vitreous (often bovine or porcine) using fluorescence-based analysis (e.g. microscopy and flow cytometry). For instance, Pitkänen *et al,* studied the cellular uptake of cationic lipoplexes to the human RPE cell line (D407) that was covered by a thin layer of bovine vitreous. They further, concluded that the vitreous substantially impedes the mobility of lipoplexes resulting in 2-30 fold decrease in the cellular uptake due to the electrostatic interactions with vitreal components [77]. In another approach, the vitreal mobility of fluorescent polystyrene nanospheres and lipoplexes and their PEGylated variants were studied using fluorescence recovery after photobleaching (FRAP) technique [205]. Nonetheless, such *in vitro* techniques involved disruption of structural network of vitreous due to experimental manipulations [76,205].

Ex vivo approaches may provide improved models of the *in vivo* condition. In this regard, Martens *et al.* and Xu *et al.* suggested an *ex vivo* bovine model to study the diffusion of drug delivery systems with single particle tracking (SPT) technique in which the mean square displacement (MSD) is determined from the microscale trajectories of particles' motion [206,207]. The data on particle motion was obtained with confocal microscopy. Unfortunately, Martens *et al*. injected the polystyrene nanospheres and polyplexes close to the anterior part of hyaloid membrane instead of central vitreous [207]. Later, single particle tracking was applied by Käsdorf *et al.* to compare the diffusion coefficient of liposomes and polystyrene nanoparticles with various charges (particle size ≈ 200 nm) in bovine, porcine and ovine vitreous [208]. Unlike the previously mentioned studies, the vitreous was not kept in the eyeball, potentially leading to alterations in the vitreous structure. In another study, the mobility of three liposome types (cationic, neutral and anionic, particle size ≈100 nm) was measured using fluorescence correlation spectroscopy (FCS) in porcine eye [209]. While single particle tracking involves live tracking of particles in the vitreous, in the FCS approach the eyes are snap frozen following the IVT injection (using 27 G needle) to study the bio-distribution of labelled nanoparticles.

In conclusion, each method has its merits and disadvantages. However, multiple factors affect the diffusion of the nanoparticles in the vitreous. For example, protein corona may be formed on the nanoparticle surface upon their exposure to the vitreous [195]. This has been investigated with a combination of surface plasmon resonance and proteomics (using mass spectrometry). This may further define the biological identity of nanoparticle and dictate their interactions with vitreal components and cells. Consequently, additional in-depth investigations are required to understand the behaviour of drug delivery systems in the vitreous.

2.8.2 Methods to study barrier role of vitreoretinal surface

The vitreoretinal (VR) interface is another important barrier hampering the retinal permeation of nanoparticles [76]. ILM is the important component of this barrier, but the precise cut-off size of the barrier has remained elusive as there are significant interspecies differences [11,96]. Similar to other barriers, ILM can reduce or block the permeation of nanoparticles into the retina depending on the physicochemical characteristics of these carriers. Positively charged nanoparticles are unable to overcome the ILM [11,96]. Even though, efficient penetration of niosomes and cationic lipid-based nanoparticles was

shown, these experiments were conducted in the rat retina [210,211] that has much thinner and less complex ILM than the larger species, like humans. Likewise, size-dependence of retinal nanoparticle penetration is not well understood, since almost all previous studies were performed in small animals with leaky and thin ILM. Consequently, *ex vivo* models may provide means to evaluate retinal permeation of nanoparticles across vitreoretinal surface. In this regard, retinal explant cultures enable studies of drug and delivery system permeation into the retina after IVT administration. Such *ex vivo* models have been widely employed to study the viral and non-viral nanoparticles such as magnetic, gold and silver nanoparticles [212-214]. However, except few studies [215,216], current knowledge is based on murine retinal explant studies [212-214,217].

Diffusion chambers have been applied to quantify drug transport across ocular membranes, such as ILM, neural retina, RPE and choroid [218-222]. In this set-up, the membrane is mounted between donor and receiver chamber, and at certain time points, permeated compound can be quantitated in the receiver phase. To date, this approach have been investigated using human, bovine, porcine and rabbit tissue membranes to study the permeation of small molecule drugs, macromolecules (e.g. bevacizumab, FITC-dextran) and nanoparticles [223]. Isolated perfused eye model is another *ex vivo* method that was first developed for biological studies, but is also applicable in pharmacokinetic studies [224]. This model has been applied in ocular studies in which ciliary artery is cannulated and perfused [224,225]. Bio-distribution of IVT injected therapeutics can be quantified in ocular tissues with mass spectrometry [226,227]. Furthermore, nanoparticle localization in ocular tissues can be detected with immunohistochemistry after cryosectioning. Perfused eye, however, is a complex method that allows only short (3-9 hours) experiments limiting its applicability in drug delivery studies [228]. A systematic research is needed to define the factors that affect retinal penetration of drug delivery systems. Such information can be further utilized in rational design of nanoparticles for retinal drug delivery.

2.8.3 Methods to study the anti-neovascularization response *in vivo*

As explained in previous sections, nvAMD is characterized by CNV. Along with the continuing attempts to introduce new treatments to suppress the neovascularization, experimental animal models are essential to screen the new therapeutics. Given the contribution of several cell types (e.g. endothelial cells and inflammatory cell) in CNV [229,230], there is no representative *in vitro* model to recapitulate the complexity of the

disease. Nonetheless, there are few reports on *ex vivo* evaluation of neovascularization based on choroidal tissue of rats or mice [231,232]. Such experimental analysis so called "choroid sprouting assay" is based on isolation of choroid from animal model and incubation in Matrigel™, in which the tube-like sprouting area is quantified to determine the anti-neovascular effects of pharmacological interventions [232]. This approach, however, requires extensive optimization to obtain robust condition while the model is applicable only for few days.

In vivo models are considered to better mimic of the neovascular disease of the patients. In this regard, CNV can be established in various animal models (e.g. rat, mouse, rabbit, pig and monkey) by means of laser photocoagulation, sub-retinal injection of VEGF or oxidative stress induction, or by applying transgenic animals to over-express the VEGF [233-235]. Currently, laser-induced rupturing of the Bruch's membrane is the most accepted preclinical CNV model [236]. In this method, newly formed blood vessels grow into the sub-retinal space mimicking the biological processes associated of nvAMD [236]. Laser-induced CNV mouse model is the most common choice for drug testing [235]. In addition to small molecule drugs, it has been applied to evaluate the efficacy of biologics, such as siRNA, recombinant proteins, antibodies, and nanoparticles [237,238]. Larger animals are better for long-term studies with CNV present for several months [239]. Larger animals also share better structural similarity with the human eye, but the cost of housing and ethical issues limit their application in drug screening [235]. Even though mouse model does not completely portray the complexity of clinical CNV pathology (e.g. absence of macula), it has helped significantly in understanding the key regulators that are involved in the progression of nvAMD. Furthermore, the model has been essential in the development of current medications, such as ranibizumab and aflibercept [236,240,241].

3 AIMS OF THE STUDY

The overall aim of this thesis was to provide better understanding of ocular barriers related to retinal drug delivery with intravitreally administered nanoparticle formulations. Such information should facilitate development of drug delivery systems for treatment of posterior segment eye diseases. This study was performed using preclinical *in vitro*, *ex vivo* and *in vivo* approaches.

The specific aims of the thesis were to:

1. Explore the intravitreal diffusion of lipid-based nanoparticles in *ex vivo* porcine model to reveal information about the relationship between particle characteristics and vitreal diffusivity.

2. Investigate the protein corona formation on liposomal surfaces in the porcine vitreous with surface plasmon resonance and mass spectrometry.

3. Investigate the role of liposome properties on their permeation from vitreous to the retinal layers.

4. Develop a liposomal drug delivery system for intravitreal sunitinib delivery and test its activity in choroidal neovascularization model *in vivo*.

4 OVERVIEW OF THE MATERIALS AND METHODS

The materials and methods applied in this thesis are summarised in Table 2. UR refers to unpublished results. Additional detailed information of the methods is shown in the original publications that are embedded to this thesis. Abbreviations of Table 2 are explained in the footnote of the table.

Table 2. Summary of materials and methods

Study	Materials and Methods	Publication
Liposome preparation and surface coating	Thin film hydration and sequential extrusion by syringe mini extruder, Avanti Polar Lipids	I, II, III, IV, UR
	DSPE-HA was synthesized using reductive amination of 8-15 kDa HA sodium salt. The conjugated lipid was used for liposome preparation with thin film hydration and extrusion.	II
	ICG loading and liposome purification with SEC using Sephadex® G-50	I, II, III
Liposome-antibody conjugation	Ramucirumab (Cyramza®) fragmentation into $F(ab')_2$ using immobilized pepsin digestion, Pierce™. $F(ab')_2$ purification with 50 kDa Pur-A-Lyzer™ dialysis membrane	UR
	Thiolated Fab' fragment was obtained by reducing the $F(ab')_2$ with 2-mercaptoethylamine hydrochloride (2-MEA) and purified with gel filtration (Superdex® 200)	UR
	Anti-VEGFR-2 Fab' fragment conjugation of liposomes using click chemistry between maleimide-PEG-PE and thiolated Fab' fragment	UR
	Targeted liposome purification from free Fab' fragments with Sepharose® CL-2B	UR
Liposome characterization	Particle size analysis with dynamic light scattering (Zetasizer APS, Malvern Instruments)	I, II, III, IV, UR
	Particle size analysis in vitreous and plasma by LALS, Apogee Flow System	II

	Surface charge (zeta potential) measurement by electro-kinetic analysis, Zetasizer ZS and DTS1070 cuvette (Malvern Instruments)	I, II, III, IV, UR
	Lipid phase transition analysis by DSC, Mettler Toledo	II
	DSPE-HA conjugation analysis by FTIR spectrometer and ^{1}H-NMR, Bruker	II
	Antibody fragmentation validation by non-reducing SDS-PAGE, BioRad™	UR
	Fab' fragment conjugation analysis by single particle automated Raman trapping analysis (SPARTA™)	
	Binding affinity to VEGFR-2 (recombinant human His-tagged protein, SinoBiological Inc.) measurement by thermophoresis, NanoTemper™	UR
Characterization of liposomal sunitinib	Sunitinib quantification and stability analysis (at 4 °C and 37 °C) by UPLC with a gradient method, Waters	IV
	Formation and characterization of sunitinib-cyclodextrin (hydroxypropyl β-cyclodextrin) inclusion complex using phase solubility method	IV
Liposome stability and drug release	ICG stability assay by light absorbance measurement through spectroscopy	II
	Passive leakage stability assay by calcein fluorescence analysis at 35-37 °C (during one week), Varioskan LUX	II
	Light-triggered release assay by calcein fluorescence analysis (Varioskan LUX) after light induction with single-mode laser module (ML6700, Modulight Inc.)	II
	Suninitib release study from liposomal formulation by rapid equilibrium dialysis, (RED device, 8 kDa cut-off), analysis by UPLC with a gradient method	IV
Biological media preparation	Porcine vitreous preparation by vitreous extraction, homogenization and filtration	I, II
	Human plasma pooling with citrate-phosphate-dextrose (CPD) anticoagulation	II, IV

Vitreal mobility study	Visualization of particles' diffusion in intact porcine vitreous within the eyecup using confocal microscopy, 3i Marianas and Slidebook 6 software	I, II
	Single particle tracking (SPT) by Imaris 9.2 software and calculation of mean square displacement (MSD) by @MSDanayzer MATLAB plugin	I, II
Protein corona structure and composition	Surface plasmon resonance measurement by MP-SPR Navi™ 200, Bionavis	I, II
	Proteomics sample preparation using in-solution tryptic digestion protocol for both plasma and vitreous	I, II
	Determination of protein concentration by BCA protein assay	I, II
	Proteomics data acquisition by LC-MS/MS, Orbitrap Fusion Instrument	I, II
	Spectral sequencing, protein groups identification and quantification in hard and soft corona with MaxQuant v.1.0.1.6	I, II
	Proteomics data analysis with principal component analysis (PCA), differential abundance and hierarchical clustering, Perseus software v. 1.5.6.0	I, II
	Computing grand average of hydropathy (GRAVY) and theoretical isoelectric point (PI) for enriched proteins in the corona by ExPASy ProtParam tool	I, II
	Additional data analysis and statistics were conducted using GeneMANIA, Venn software and GraphPad Prism	I, II
	Protein corona thickness measurement by modified Jung model, Spreadsheet software	I, II
Ex vivo retinal penetration study	Preparation of bovine retinal explants (with and without vitreous) and culturing on Transwell® nourished by supplemented Neurobasal® A-medium	III
	Retinal explant exposure to liposomes through direct application on top of the retina or intravitreal injection using 30 G needle	III
	Tissue snap freezing using dry ice following the cryopreservation protocol (30 % sucrose)	III

	Cryo-sectioning from different retinal explant sites with Cryostat, Leica CM3050s	III
	Immunohistochemistry using rabbit anti-collagen IV antibody (1:200), Alexa Fluor™ 488-tagged goat anti-rabbit secondary antibody (1:500) and Hoechst (1:1000), Invitrogen	III
	Visualization and imaging performed with a confocal microscope, Leica TCS SP8	III
In vivo anti-neovascularization study	Preparation of laser-induced choroidal neovascularization mouse model by retinal photocoagulation laser, Vitra2 Quantal Medical	IV
	Formation and development of CNV was monitored using optical coherence tomography (OCT) and fluorescein fundus angiography (FA) (Micron IV; Phoenix Research Labs	IV
	Comparative analysis of the leakage area following the animal treatment using angiograms	IV
Additional analysis	Image analysis and statistical analysis by FIJI, ImageJ 1.51 and GraphPad Prism 8.2.1	III, IV

HA, hyaluronic acid; **DSPE**, 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine; **ICG**, indocyanine green; **SEC**, size exclusion chromatography; **VEGFR-2**, vascular endothelial growth factor receptor-2; **LALS**, large angle light scattering; **DSC**, differential scanning calorimetry; **FTIR**, Fourier-transform infrared spectroscopy; **^{1}H-NMR**, proton nuclear magnetic resonance; **SDS-PAGE**, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; **UPLC**, ultra-performance liquid chromatography; **BCA**, bicinchoninic acid assay; **LC-MS/MS**, liquid chromatography-tandem mass spectrometry.

5 PUBLICATION I: Diffusion and Protein Corona Formation of Lipid-based Nanoparticles in Vitreous Humor: Profiling and Pharmacokinetic Considerations

Reprinted with permission from Tavakoli S, Kari O.K, Turunen T, Lajunen T, Schmitt M, Lehtinen J, Tasaka F, Parkkila P, Ndika J, Viitala T, Alenius H, Urtti A, Subrizi A: Diffusion and protein corona formation of lipid-based nanoparticles in vitreous humor: Profiling and pharmacokinetic considerations. *Molecular Pharmaceutics* 18 (2): 699-713, 2020. © American Chemical Society 2020.

Available online at: https://pubs.acs.org/doi/abs/10.1021/acs.molpharmaceut.0c00411

6 PUBLICATION II: Light-Activated Liposomes Coated with Hyaluronic Acid as a Potential Drug Delivery System.

Reprinted with permission from Kari O.K, Tavakoli S, Baan S, Savolainen R., Ruoslahti T., Johansson N.G., Ndika J., Alenius H., Viitala T., Urtti A., Lajunen T.: Light-activated liposomes coated with hyaluronic acid as a potential drug delivery system. *Pharmaceutics* 12(8): 763, 2020. © MDPI 2020.

Available online at: https://www.mdpi.com/1999-4923/12/8/763

7 PUBLICATION III: Ocular barriers to retinal delivery of intravitreal liposomes: Impact of vitreoretinal interface.

Reprinted with permission from Tavakoli S., Peynshaert K., Lajunen T., Devoldere J., del Amo E., Ruponen M., De Smedt S., Remaut K., Urtti A.: Ocular barriers to retinal delivery of intravitreal liposomes: Impact of vitreoretinal interface. *Journal of Controlled Release* 328: 952-961, 2020.

© Elsevier 2020.

Available online at: https://www.sciencedirect.com/science/article/pii/S0168365920306064

8 PUBLICATION IV: Liposomal sunitinib in ocular drug delivery: A potential treatment for choroidal neovascularization

Unpublished manuscript. © Authors 2021

10 DISCUSSION

Intravitreal VEGF-neutralizing biologics are the main treatment for the retinal and choroidal diseases (e.g. AMD and DR). However, it is now known that other mechanisms are also involved in the pathogenesis of these diseases, including inflammation and neurodegeneration that are not responsive to anti-VEGF therapy [118]. Small molecule drugs show promise in the retinal and choroidal treatment [5,57], but their elimination from the vitreous is fast (half-lives 1-10 hours), resulting in poor clinical utility as intravitreal solutions [4,80]. Therefore, prolongation of vitreal drug retention and sustained release are required formulation properties. In this respect, one approach is to employ nanotechnology-based drug delivery systems for prolonged retention in the eye. Despite all efforts, retinal drug delivery is still challenging due to the complex ocular barriers. This thesis provides information on the fundamentals that must be considered in the design of retinal drug delivery systems based on lipid-based nanoparticles. In the following sections, the methods and results of this study are discussed.

10.1. Mobility of lipid-based nanoparticles in the vitreous

The vitreous humor is the first barrier that nanoparticles encounter upon IVT injection. The interaction of nanoparticles with the vitreous may have influence on their ocular pharmacokinetics. Publication **I** and **II** involved characterization of nanoparticles in physiological environment and evaluation of nanoparticle diffusion in the vitreous.

Rodents have impaired translational value due to the significant anatomical differences compared to the human eye. Therefore, this study was based on *ex vivo* investigations of intact porcine vitreous. Mobility of nanoparticles was analysed using a modified procedure based on the report of Xu and colleagues [206]. The relevance of animal model is of great importance, since the size of the eye (vitreous volume) and the vitreous structure can affect the nanoparticle mobility. In this experimental system, the anterior segment of the porcine eye was removed, exposing the intact vitreous. Diffusion of labelled nanoparticles were followed after IVT injections into the centre of vitreous gel. The average anteroposterior axis in porcine eye (23.9 mm) is similar with the human eye (24 mm) [242]. Furthermore, the viscoelastic properties of central porcine vitreous are similar to human vitreous [243-

246], whereas bovine vitreous has higher viscosity [245,247]. Also, the concentration of HA in the central porcine vitreous and human vitreous are similar; important factor for the the electrostatic interactions between vitreous and nanoparticles [248]. This improved our confidence on the translational value of our *ex vivo* model, even though most of earlier studies on vitreal mobility have utilized bovine vitreous [206,207].

Single particle tracking was applied in this study allowing determination of the mean square displacement and diffusion coefficients of labelled IVT nanoparticles [206,207]. Single particle tracking allows live tracking of nanoparticles at high temporal and spatial resolution [249]. This method has been used in colloidal studies and biophysics and it enables discrimination between random diffusion, convection and obstructed movements in the matrix [249,250]. Such analyses provide valuable information regarding the extent of vitreous impediment to nanoparticles' movement. Furthermore, it informs about the influence of nanoparticle properties on vitreal mobility. Therefore, we compared the vitreal mobility of 36 IVT liposomal formulations with different sizes, surface coatings and charges. Also other lipid-based nanoparticles, such as hexosomes and NLCs, were assessed with the same method.

Overall, our work shows that neutral and negatively charged lipid-based nanoparticles (< 200 nm) are relatively freely mobile in the vitreous (Fig. 2, publication **I**), but the vitreous severely obstructs diffusion of the cationic formulations regardless of their size. This highlights the significant role of surface charge in the mobility of nanoparticles in the vitreous. In terms of surface coating, we observed that the effect of surface coating is more prominent in less mobile nanoparticles (e.g. cationic liposomes), while it showed marginal to moderate impact on the diffusion of anionic and neutral particles. Presumably, PEG and HA can mask the cationic charges on the liposomal surface to some extent thereby reducing the electrostatic interactions, but the diffusion was still more restricted than in the case of anionic and neutral liposomes.

Interestingly, the data shows that PEG-coated liposomes diffuse faster than the HA-coated liposomes. Steric shielding of PEG seems to interact less than HA with the vitreous (Fig. 10, publication **II**). Hence, negative charges (HA) do not necessarily improve the vitreal mobility over neutral coating (PEG) suggesting that the interplay with the vitreous is complex and involves many mechanisms. Furthermore, the high-resolution proteomics study confirmed that the HA-coated liposomes interact with structural collagen meshwork

that may render them less mobile (publication **II**). This feature may be employed to extend the vitreal half-life by HA-conjugation. For instance, HA-conjugated sFlt-1 (soluble VEGF decoy receptor) had 10-fold longer vitreal half-life than the unconjugated sFlt-1 [251]. Although, the authors linked this behaviour to the increased molecular size, one should note that the interaction of HA with the vitreal components may also contribute to improved ocular retention. Similarly, ocular retention and duration of action of IVT nanoparticles might be improved by HA coating. Huang *et al.* reported prolonged retinal effect in rat model after coating Cx43-mimetic peptide containing human serum albumin with HA [252]. Herein, the HA was applied to target the CD44-receptor expressing retinal cells, but the extended vitreal retention can be related to HA-collagen interactions.

Our data and calculations suggest that the vitreal distribution of nanoparticles *in vivo* is probably controlled by convection in healthy young vitreous, but it may shift toward diffusion-control with aging as the vitreous undergo liquefaction (Table 3, publication **I**). On the contrary, the effect of vitreal liquefaction on the mobility of antibodies is inconsequential, since their transport is mostly governed by diffusion. The vitreal mobility behaviour in the vitreous substitutes, however, remains elusive, because the velocity of convective flow and stability of nanoparticles in vitrectomized eyes are not known. Furthermore, it seems that in small rodent eyes diffusion is always the major controlling factor in the IVT distribution of drugs and nanoparticles.

Moreover, pharmacokinetics of liposomes and other nanoparticles may be influenced by formation of biocorona or protein corona on their surface. Thus, the characteristics of nanoparticles may change after their exposure to the vitreous [253]. This biological identity may shape cell level pharmacological properties of the nanoparticles as well as their vitreal mobility. Although, the importance of protein corona has been recognized in nanomedicine [253,254], the vitreal corona of liposomes has not been previously explored [255,256]. Protein interactions may also affect drug release behaviour and cellular uptake of liposomes [257]. Therefore, we explored the protein corona formation on the liposomes in the presence of porcine vitreous using recently established work flow that involves sequential surface plasmon resonance and mass spectrometry analyses of the protein coronas. We explored uncoated, PEGylated and HA-coated light activated liposomes (Table 4, publication **II**). The extent of protein corona formation was comparable between anionic PEGylated (50 nm) and uncoated liposomes indicating that the PEGylation did not

attract more vitreal proteins. Even though protein corona increased the liposome size by 10-12%, it is likely that their vitreal mobility was not significantly affected. The same conclusion is applicable also to HA-coated liposomes, even though they bound more protein than the PEG-coated liposomes. In addition, our observations suggest that the anionic liposomes retain their negative charge in the vitreous irrespective of their coating, since the twenty most abundant proteins in the liposomal protein coronas were predominately negatively charged (Fig. 7, publication **II**). Hence, it is evident that protein corona does not mask the negative surface charge of the liposomes.

The comprehensive analysis of protein properties revealed the presence of immune system components (e.g. complement C3, clusterin, apolipoprotein E) in the protein corona of anionic and neutral liposomes implying that the opsonisation may be involved in the ocular elimination process.

Nonetheless, further work is required to elucidate the impact of protein corona on ocular biodistribution of liposomes in healthy and diseased eyes. The findings build improved understanding of the barriers in retinal drug delivery.

10.2 Retinal penetration of liposomes across the vitreoretinal interface

In publication **I** and **II**, we demonstrated the key factors affecting the mobility of liposomes in the vitreous. However, in many cases liposomes must distribute to the retina to deliver their cargoes into the retinal cells in order to exert actions of RNA, DNA or intracellularly acting peptides in the cells. Therefore, after successful distribution within the vitreous, the liposomes should permeate into the retina across the vitreoretinal barrier (in particular, ILM). Therefore, we explored retinal permeation of anionic and neutral liposomes with good vitreal mobility. Moreover, we studied the effects of PEG coating, surface charge, and size of liposomes on retinal penetration (publication III).

There are many published reports on effective retinal drug delivery with nanoparticles, but the experiments were performed in murine *in vivo* or *ex vivo* models [210,211,258]. As discussed above, ocular barriers are different in small laboratory animals compared to larger species and humans. Thus, we used bovine retinal explants to obtain relevant information about ILM barrier. The thickness of bovine ILM is comparable to the human ILM, whereas the rodents have simpler and thinner ILM structure that is more similar to

the human foetus (ILM thickness ≈ 0.07 μm) [87,88]. In addition, the ILM undergoes age-related changes leading to thicker and stiffer structure [86]. These facts highlight the importance of relevant models. Herein, two different retinal explants were used: without vitreous (R-explant) and with attached vitreous (VR-explant). The latter model simulates the structure of vitreoretinal interface and allows to explore the role of the intact ILM, but the VR-explant preparation is more laborious than the R-explant. Therefore, we first evaluated the penetration of liposomes in the R-explant and, later, selected cases in VR-explant.

In R-explant, the eyecup was cut to have 4 flaps, optic nerve being in one of the flaps, but the optic nerve region was avoided in the experiments due to its different structure [11]. The other three flaps were punched using similar distances from the centre of the eyecup and treated with optimized volume of liposomes (10 μl) ensuring that the formulation covers the explant but without over-spilling to the edges of the explants. In VR-explant, the liposomes were injected IVT in the centre of explant (Fig. S1, publication **III**) and the needle was taken out of the vitreous after few seconds to avoid spillage of liposomes due to backpressure. We also made sure not to take any microscopic images at the edges of the retinal sections. With such considerations, we minimized the risk of misleading results due to any edge contact of the liposomes.

Overall, the small anionic liposomes (< 50 nm) with PEG showed the best retinal distribution. The semi-quantitative analysis helped us to highlight the impact of surface charge in retinal entry. According to Figure 2C (publication **III**), the number of sections with more than 42 particles (data above 75th percentile) inside the retinal layers was higher for anionic liposomes than for neutral ones. Presumably, the anionic liposomes have lower interactions than neutral liposomes both with vitreous and ILM. In addition, the Müller cells may affect retinal penetration due to their interactions with anionic particles as they are phagocytic cell type [216]. In terms of surface coating, PEG displayed positive impact on liposomal permeation across the vitreoretinal barrier. This was seen as more consistent retinal penetration of PEGylated liposomes as compared to uncoated ones. Even though there are sparse data about influence of PEG on retinal permeation, a recent study reported that PEGylation with a molecular weight of ≥ 2000 Da improves the nanoparticle transport across ocular mucosal barriers [259]. This can also be leveraged to explain higher retinal entry of PEG-coated liposomes, because the structural components of vitreous and ILM are

relatively similar with the mucus. Interestingly, co-localization of liposomes with retinal ganglion cells (RGC) was observed, implying potential application of the localization for targeting RGC to treat retinal degeneration in glaucoma [39,52] (Fig. 1, publication **III**).

In addition to surface charge and surface coating, retinal permeation is also influenced by particle size as larger liposomes (≈100 nm) failed to cross the vitreoretinal barrier. This suggests that the sieve-like structure of ILM does not allow particles of over 100 nm to enter retinal layers. Nonetheless, breached ILM displayed leakier barrier in which larger liposomes distributed in retinal layers (Fig. S3, publication **III**). Similarly, in certain diseases, such as proliferative diabetic retinopathy, the ILM may become compromised potentially leading to higher retinal permeation, but the leakiness is often associated with late-stage of disease [260]. The relation between retinal distribution and disease state is complex and it is more meaningful to study the barrier role firstly in the intact ILM. This would help to design a drug delivery system that permeate into the retina even through the uncompromised vitreoretinal interface.

Our data extends understanding of the retinal barriers, which is important in the design of drug and gene delivery systems for retinal therapy. Indeed, retinal barriers determine the route of administration, for instance, subretinal injection can be considered the most effective method for delivering of viral vectors since their permeation from vitreous into the retina is challenging. Nonetheless, problems in understanding the barriers and use of rodents in the preclinical studies have generated over-optimistic expectations, partly explaining the lack of IVT nanoparticles and viral vectors in the clinical use.

10.3 Liposomal sunitinib for treatment of choroidal neovascularization

Study IV involved development of sunitinib-loaded liposomes for treatment of choroidal neovascularization (CNV). Sunitinib maleate has received FDA approval for oral administration in the treatment of renal cell carcinoma [261,262]. It is a tyrosine kinase inhibitor which blocks all VEGFRs as well as PDGFR leading to suppression of the VEGF expression in newly formed choroidal vessels [5,140,142,263-265]. Thus, it is an alternative approach to anti-VEGF biologics for blocking the VEGF signalling pathway associated with ocular neovascularization. In addition to the ocular neovascularization,

sunitinib may be useful in ocular diseases characterized with retinal degeneration (such as glaucoma and dry-AMD) owing to its neuroprotective properties [137].

Considering the dose-dependent toxicity of sunitinib and the rapid intravitreal elimination of small molecules, a drug delivery system is required for intraocular administration of sunitinib [4,57]. With this potential in mind, we developed anionic PEGylated (≈ 100 nm) liposomes through thin film hydration method for solubilizing the sunitinib as IVT injectable formulation. Liposomal formulations are biocompatible and biodegradable, and they have been accepted for clinical use as eye drops and intravenous injections [266,267]. For example, Visudyne® is an intravenous liposomal photodynamic therapy product for CNV treatment [8]. IVT formulation would avoid the systemic toxicity of sunitinib by low dose and limiting drug release to the intraocular space.

Anionic liposomes with PEG coating are capable to overcome relevant ocular barriers, as discussed in previous sections. Hence, lipid composition was selected accordingly, including DSPG and PEG-DSPE in the formulation. Unlike light-activated liposome formulation, in which lower phase transition temperature was desired, we substituted the single hydrocarbon chain phospholipid (Lyso-PC) and short length hydrocarbon chain phospholipid (DPPC) with DSPC and DOPC. Such a composition avoids lipid bilayer leakiness while it provides sufficient firmness to prolong the drug release for a few days (Fig. 2, **IV**). In this study, we used sunitinib as lipophilic free base form to maximize encapsulation and optimize drug release. In this regard, sunitinib was encapsulated in the lipid bilayer by passive loading method that resulted in 95 % encapsulation efficiency. Also, liposomal sunitinib revealed relatively high loading capacity (≈ 5 %) allowing intravitreal dosing of at least 1 µg per 1 µl of injection [268]. Although encapsulation of sunitinib results in promising encapsulation efficiency, evaluation of its release profile from liposomes was challenging, because it has poor water-solubility. We addressed the solubility issue by introducing cyclodextrin to the receiver chamber of equilibrium dialysis device. In this respect, the solubility of sunitinib in PBS was improved up to 150-fold when hydroxypropyl β-cyclodextrin was complexed with sunitinib at 24:1 molar ratio. Herein, excess amount of hydroxypropyl β-cyclodextrin, a FDA-approved excipient for ophthalmic preparations, was employed to form inclusion complexes with sunitinib (Fig. 1, **IV**) thereby providing sink conditions for drug release.

The formulation was studied first for endotoxin with Limulus Amoebocyte Lysate (LAL) assay, which confirmed the non-pyrogenic injection material. The CNV mice received 1 µl of IVT liposomal sunitinib. It was evident from our fluorescein angiograms that liposomal sunitinib effectively prevented the vascular leakage for 3 days, presumably by reducing drug elimination from the vitreous while sunitinib-cyclodextrin solution did not show any anti-neovascular effect. Furthermore, evidence indicates that the ocular pharmacokinetics of sunitinib can be influenced by its binding to melanin in pigmented tissues, such as iris, ciliary body, RPE and choroid [269]. In such conditions, the drug-melanin complex may lead to reduced concentration of free drug, possibly reducing the peak pharmacological response [270]. Nevertheless, melanin complex may also serve as a drug depot thus extending the half-life of drug in the posterior segment of the eye [271]. Moreover, the retention time is generally longer when we translate it to human eye owing to its larger volume of the vitreous (4 ml) compared to mouse model (5 µl).

Overall, sunitinib offers promising therapeutic option for treatment of nvAMD owing to its dual role of being neuroprotective and anti-neovascular compound. Previously, we discussed about the survival role of sunitinib for retinal ganglion cells by inhibition of dual leucine zipper kinase [137]. Although the mechanism of sunitinib protection of the photoreceptors is not yet known, Tsujinaka and colleagues observed thickening of the outer nuclear layer with sunitinib therapy in CNV mice, suggesting that sunitinib is involved in photoreceptor survival [264]. Therefore, sunitinib may pose an advantage over current anti-VEGF biologics in treatment of nvAMD. In this regards, Grunwald and colleagues revealed that long-term monthly injection of ranibizumab is a risk factor for photoreceptor cell death as 18% of patients on ranibizumab treatment developed GA after 2 years [272].

10.4 Future prospects

In this work, we demonstrated that multiple factors are involved in successful retinal drug delivery with liposomes. However, many other aspects have yet to be studied regarding the correlation between liposome properties and their efficacy in circumventing ocular barriers. Herein, we applied healthy juvenile vitreous for diffusion study of lipid-based nanoparticles, but additional work is required to study their diffusion in diseased state models with more liquid vitreous, vitrectomized eyes and vitreous substitutes (e.g. silicon

oil and polymeric hydrogels) to assess behavior of liposomes in those conditions. Even though there are some reports on vitreal clearance of anti-VEGF drugs (ranibizumab and aflibercept) in vitrectomized non-human primates such as macaque eyes [273,274], the knowledge about the effect of vitrectomy on vitreal distribution of particulate formulations is limited. Therefore, further investigation is needed to understand the factors that affect vitreal mobility (diffusion and convection) in various conditions. Certainly, improved tools and understanding are needed for inter-species translation of *in vivo* data to the humans [275,276].

The protein binding interactions may also be influenced in diseased eyes, thus it would be useful to explore the protein corona enrichment using a diseased vitreous involving diabetic changes [277]. In this case, the vitreal proteins may be different, potentially affecting ocular pharmacokinetics of liposomes. Furthermore, the impact of different protein classes on immune response, safety and cellular interactions of liposomes requires further exploration.

Herein, the fundamentals for efficient transport of nanoparticles from the site of intravitreal injection toward retina were presented, nonetheless another way to leverage these information would be to develop less mobile formulations for prolonged ocular retention and sustained drug release. This can be achieved, for instance, by HA-modification of liposomes to increase the interactions with collagen network, although optimization of the HA coating parameters is still needed. Alternatively, increasing the size of nanoparticles above the mesh size of vitreous meshwork (> 550 nm) may provide extended therapeutic concentrations, if drug release will be slow enough and adequate drug loading can be accomplished. Such approach could be combined with melanin-binding drugs, such as sunitinib, to generate a secondary depot in pigmented eye tissues for further prolongation of drug effects. GB-102 formulation (sunitinib loaded polymeric-based microparticles) is an example of this strategy, and is currently in clinical trials for wet-AMD with twice-yearly dosing regimen [140].

Our findings confirmed that removal of the vitreous, common procedure in conventional explant studies, and use of mice retina have led to overestimation of nanoparticles' retinal distribution. We presented a bovine vitreoretinal explant model that is more representative for human situation. The model is in line with three R principles, allowing simultaneously investigating nanoparticle interactions with vitreous and ILM, the main barriers in retinal

drug delivery. Nonetheless, this model could be improved by dynamic circulation of culture medium in flow systems (e.g. Quasi-vivo®).

As discussed earlier, physicochemical characteristics of lipid-based nanoparticles influence their retinal permeation. Numerous biological factors, including age, disease and cellular activity, may be involved in retinal distribution, but are still unknown. In addition localization of small anionic PEGylated liposomes was observed in ganglion cell layers, it is therefore of great interest to explore the potential of such formulations for the delivery of neuroprotective compounds for treatment of retinal neurodegenerative conditions, such as glaucoma. Interestingly, we have observed that the shape (tubular vs spherical) of polymeric nanostructure seems to affect their ability to pass vitreoretinal interface [278], but more detailed studies are still needed.

Drug treatment may be affected by cellular resistance mechanisms, such as influx and efflux transport [279]. For instance, sunitinib is a substrate for P-glycoprotein transport in the RPE cells. Interestingly, nanosystems may bypass efflux transport within the cells, thus improving drug efficacy. Taken together, although the anti-VEGF injections revolutionized the treatment of retinal diseases, such as AMD, further improvements are needed to enable use of intracellular biologics (e,g RNA) in retinal therapy, to prolong the injection intervals of intravitreal drugs and targeting drugs to the retinal and choroidal cells.

11 CONCLUSIONS

This thesis is focused on the properties of lipid-based nanoparticles, such as liposomes, as retinal drug delivery systems. The following specific conclusions were reached.

1. Surface charge of nanoparticles has significant impact on their vitreal mobility, cationic particles showing much lower mobility than the anionic and neutral ones. Increasing size of the lipid-based nanoparticles (from < 50 nm up to 200 nm) has modest slowing effect of mobility when the particle size was smaller than pore size of the vitreous meshwork.

2. PEG-coating improves the vitreal diffusion of liposomes, particularly in the case of slowly diffusing cationic liposomes.

3. HA-coated liposomes have lower vitreal mobility than PEGylated counterparts due to their interactions with vitreal components.

4. IVT distribution of nanoparticles is dominated by convection in large eyes, whereas antibody distribution is diffusion-dependent. In rodent eyes, vitreal distribution is ruled by diffusion.

5. Protein corona formation after exposure to porcine vitreous affects liposomes size only minimally, thereby not affecting their mobility in the vitreous. Liposome coating with PEG or HA do not result in further protein enrichment.

6. Vitreoretinal interface in bovine retinal explants is a significant barrier for retinal penetration of liposomes and serves as valuable model for retina permeation studies.

7. Liposomes over 100 nm fail to overcome vitreoretinal interface barrier, while small liposomes below 50 nm in diameter do permeate to the retina.

8. Negative surface charge and PEG-coating facilitate the retinal permeation of liposomes.

9. Liposomes are capable of localization to the retinal ganglion cells.

10. Anionic PEGylated liposomes efficiently encapsulate sunitinib and showed anti-neovascular effect in CNV mouse model suggesting potential of IVT liposomal sunitinib treatment.

REFERENCES

[1] S.R. Flaxman, R.R.A. Bourne, S. Resnikoff, P. Ackland, T. Braithwaite, M. V. Cicinelli, A. Das, J.B. Jonas, J. Keeffe, J. Kempen, J. Leasher, H. Limburg, K. Naidoo, K. Pesudovs, A. Silvester, G.A. Stevens, N. Tahhan, T. Wong, H. Taylor, A. Arditi, Y. Barkana, B. Bozkurt, A. Bron, D. Budenz, F. Cai, R. Casson, U. Chakravarthy, J. Choi, N. Congdon, R. Dana, R. Dandona, L. Dandona, I. Dekaris, M. Del Monte, J. Deva, L. Dreer, L. Ellwein, M. Frazier, K. Frick, D. Friedman, J. Furtado, H. Gao, G. Gazzard, R. George, S. Gichuhi, V. Gonzalez, B. Hammond, M.E. Hartnett, M. He, J. Hejtmancik, F. Hirai, J. Huang, A. Ingram, J. Javitt, C. Joslin, M. Khairallah, R. Khanna, J. Kim, G. Lambrou, V.C. Lansingh, P. Lanzetta, J. Lim, K. Mansouri, A. Mathew, A. Morse, B. Munoz, D. Musch, V. Nangia, M. Palaiou, M.B. Parodi, F.Y. Pena, T. Peto, H. Quigley, M. Raju, P. Ramulu, D. Reza, A. Robin, L. Rossetti, J. Saaddine, M. Sandar, J. Serle, T. Shen, R. Shetty, P. Sieving, J.C. Silva, R.S. Sitorus, D. Stambolian, J. Tejedor, J. Tielsch, M. Tsilimbaris, J. van Meurs, R. Varma, G. Virgili, Y.X. Wang, N.L. Wang, S. West, P. Wiedemann, R. Wormald, Y. Zheng, Global causes of blindness and distance vision impairment 1990–2020: a systematic review and meta-analysis, Lancet Glob. Heal. 5 (2017) e1221–e1234. doi:10.1016/S2214-109X(17)30393-5.

[2] Y.C. Tham, X. Li, T.Y. Wong, H.A. Quigley, T. Aung, C.Y. Cheng, Global prevalence of glaucoma and projections of glaucoma burden through 2040: A systematic review and meta-analysis, Ophthalmology. 121 (2014) 2081–2090. doi:10.1016/j.ophtha.2014.05.013.

[3] A. Urtti, Challenges and obstacles of ocular pharmacokinetics and drug delivery, Adv. Drug Deliv. Rev. 58 (2006) 1131–1135. doi:10.1016/j.addr.2006.07.027.

[4] E.M. del Amo, A.K. Rimpelä, E. Heikkinen, O.K. Kari, E. Ramsay, T. Lajunen, M. Schmitt, L. Pelkonen, M. Bhattacharya, D. Richardson, A. Subrizi, T. Turunen, M. Reinisalo, J. Itkonen, E. Toropainen, M. Casteleijn, H. Kidron, M. Antopolsky, K.S. Vellonen, M. Ruponen, A. Urtti, Pharmacokinetic aspects of retinal drug delivery, Prog. Retin. Eye Res. 57 (2017) 134–185. doi:10.1016/j.preteyeres.2016.12.001.

[5] N. Ferrara, A.P. Adamis, Ten years of anti-vascular endothelial growth factor therapy, Nat. Rev. Drug Discov. 15 (2016) 385–403. doi:10.1038/nrd.2015.17.

[6] N. Waugh, E. Loveman, J. Colquitt, P. Royle, J.L. Yeong, G. Hoad, N. Lois, Treatments for dry age-related macular degeneration and stargardt disease: A systematic review, Health Technol. Assess. (Rockv). 22 (2018) 1–167. doi:10.3310/hta22270.

[7] R. Bisht, A. Mandal, J.K. Jaiswal, I.D. Rupenthal, Nanocarrier mediated retinal drug delivery: overcoming ocular barriers to treat posterior eye diseases, Wiley Interdiscip. Rev. Nanomedicine Nanobiotechnology. 10 (2018) e1473. doi:10.1002/wnan.1473.

[8] U.B. Kompella, A.C. Amrite, R.P. Ravi, S.A. Durazo, Nanomedicines for back of the eye drug delivery, gene delivery, and imaging, (2013). doi:10.1016/j.preteyeres.2013.04.001.

[9] O.K. Kari, J. Ndika, P. Parkkila, A. Louna, T. Lajunen, A. Puustinen, T. Viitala, H. Alenius, A. Urtti, In situ analysis of liposome hard and soft protein corona structure and composition in a single label-free workflow, Nanoscale. 12 (2020) 1728–1741.

[10] N. Weng, W. Wei, X. Zhu, The morphologic structure of vitreous hemorrhage and posterior vitreous detachment, Chung Hua Yen Ko Tsa Chih. 37 (2001) 425–427. http://www.ncbi.nlm.nih.gov/htbin post/Entrez/query?db=m&form=6&dopt=r&uid=11840748.

[11] K. Peynshaert, J. Devoldere, A.K. Minnaert, S.C. De Smedt, K. Remaut, Morphology and Composition of the Inner Limiting Membrane: Species-Specific Variations and Relevance toward Drug Delivery Research, Curr. Eye Res. 44 (2019) 465–475. doi:10.1080/02713683.2019.1565890.

[12] K. Halasz, Y. V. Pathak, Liposomes for retina and posterior segment disease, in: Drug Deliv. Retin. Posterior Segm. Dis., Springer International Publishing, 2018: pp. 97–106. doi:10.1007/978-3-319-95807-1_6.

[13] S. Lai, Y. Wei, Q. Wu, K. Zhou, T. Liu, Y. Zhang, N. Jiang, W. Xiao, J. Chen, Q. Liu, Y. Yu, Liposomes for effective drug delivery to the ocular posterior chamber, J. Nanobiotechnology. 17 (2019). doi:10.1186/s12951-019-0498-7.

[14] Eric Pearlman John V. Forrester, Andrew D. Dick, The Eye: Basic science in practice, 4th ed., Elsevier, 2016. doi:10.1016/C2012-0-07682-X.

[15] H. Qiao, T. Hisatomi, K.H. Sonoda, S. Kura, Y. Sassa, S. Kinoshita, T. Nakamura, T. Sakamoto, T. Ishibashi, The characterisation of hyalocytes: The origin, phenotype, and turnover, Br. J. Ophthalmol. 89 (2005) 513–517. doi:10.1136/bjo.2004.050658.

[16] W. Halfter, U. Winzen, P.N. Bishop, A. Eller, Regulation of eye size by the retinal basement membrane and vitreous body, Investig. Ophthalmol. Vis. Sci. 47 (2006) 3586–3594. doi:10.1167/iovs.05-1480.

[17] M.M. Le Goff, P.N. Bishop, Adult vitreous structure and postnatal changes, Eye. 22 (2008) 1214–1222. doi:10.1038/eye.2008.21.

[18] J. Sebag, Age-related changes in human vitreous structure, Graefe ' s Arch. Ophthalmol. 225 (1987) 89–93. doi:10.1007/BF02160337.

[19] A. 1 B. Bristol, Structure, Function and Ageing of the Collagens of the Eye, 1987. doi:10.1038/eye.1987.34.

[20] E.M. Laicine, A. Haddad, Transferrin one of the major vitreous proteins, is produced within the eye, Exp. Eye Res. 59 (1994) 441–446. doi:10.1006/exer.1994.1129.

[21] M. Angi, H. Kalirai, S.E. Coupland, B.E. Damato, F. Semeraro, M.R. Romano, Proteomic analyses of the vitreous humour, Mediators Inflamm. 2012 (2012). doi:10.1155/2012/148039.

[22] J.N. Ulrich, M. Spannagl, A. Kampik, A. Gandorfer, Components of the fibrinolytic system in the vitreous body in patients with vitreoretinal disorders, Clin. Exp. Ophthalmol. 36 (2008) 431–436. doi:10.1111/j.1442-9071.2008.01793.x.

[23] J.U.C.W.M.S.A.K.A. Gandorfer, Components of the Fibrinolytic System in the Vitreous Body in Patients With Vitreoretinal Disorders | IOVS | ARVO Journals, Clin. Exp. Ophthalmol. Exp Ophthalmol . 36 (2008) 431–436. https://iovs.arvojournals.org/article.aspx?articleid=2394260 (accessed December 14, 2020).

[24] A.-K. Rimpelä, S. Reunanen, M. Hagströ, H. Kidron, A. Urtti, Binding of Small Molecule Drugs to Porcine Vitreous Humor, (2018). doi:10.1021/acs.molpharmaceut.8b00038.

[25] T.L. Jackson, R.J. Antcliff, J. Hillenkamp, J. Marshall, Human retinal molecular weight exclusion limit and estimate of species variation, Investig. Ophthalmol. Vis. Sci. 44 (2003) 2141–2146. doi:10.1167/iovs.02-1027.

[26] E. Ramsay, M. Hagström, K.S. Vellonen, S. Boman, E. Toropainen, E.M. del Amo, H. Kidron, A. Urtti, M. Ruponen, Role of retinal pigment epithelium permeability in drug transfer between posterior eye segment and systemic blood circulation, Eur. J. Pharm. Biopharm. 143 (2019) 18–23. doi:10.1016/j.ejpb.2019.08.008.

[27] F. Germain, J. Vicente, Functional histology of the retina, Undefined. (2010).

[28] V.M. Reddy, R.L. Zamora, R.J. Olk, Quantitation of retinal ablation in proliferative diabetic retinopathy, Am. J. Ophthalmol. 119 (1995) 760–766. doi:10.1016/S0002-9394(14)72782-5.

[29] P.A. Campochiaro, Ocular neovascularization., J. Mol. Med. (Berl). 91 (2013) 311–321. doi:10.1007/s00109-013-0993-5.

[30] K.M. Hämäläinen, K. Kananen, S. Auriola, K. Kontturi, A. Urtti, Characterization of paracellular and aqueous penetration routes in cornea, conjunctiva, and sclera, Investig. Ophthalmol. Vis. Sci. 38 (1997) 627–634.

[31] E.M. del Amo, A. Urtti, Rabbit as an animal model for intravitreal pharmacokinetics: Clinical predictability and quality of the published data, Exp. Eye Res. 137 (2015) 111–124. doi:10.1016/j.exer.2015.05.003.

[32] J.M. Ramírez, A.I. Ramírez, J.J. Salazar, R. De Hoz, A. Trivio, Changes of astrocytes in retinal ageing and age-related macular degeneration, Exp. Eye Res. 73 (2001) 601–615. doi:10.1006/exer.2001.1061.

[33] J.M. Seddon, J. Cote, N. Davis, B. Rosner, Progression of age-related macular degeneration: Association with body mass index, waist circumference, and waist-hip ratio, Arch. Ophthalmol. 121 (2003) 785–792. doi:10.1001/archopht.121.6.785.

[34] R.R.A. Bourne, S.R. Flaxman, T. Braithwaite, M. V. Cicinelli, A. Das, J.B. Jonas, J. Keeffe, J. Kempen, J. Leasher, H. Limburg, K. Naidoo, K. Pesudovs, S. Resnikoff, A. Silvester, G.A. Stevens, N. Tahhan, T. Wong, H.R. Taylor, P. Ackland, A. Arditi, Y. Barkana, B. Bozkurt, R. Wormald, A. Bron, D. Budenz, F. Cai, R. Casson, U. Chakravarthy, N. Congdon, T. Peto, J. Choi, R. Dana, M. Palaiou, R. Dandona, L. Dandona, T. Shen, I. Dekaris, M. Del Monte, J. Deva, L. Dreer, M. Frazier, L. Ellwein, J. Hejtmancik, K. Frick, D. Friedman, J. Javitt, B. Munoz, H. Quigley, P. Ramulu, A. Robin, J. Tielsch, S. West, J. Furtado, H. Gao, G. Gazzard, R. George, S. Gichuhi, V. Gonzalez, B. Hammond, M.E. Hartnett, M. He, F. Hirai, J. Huang, A. Ingram, C. Joslin, R. Khanna, D. Stambolian, M. Khairallah, J. Kim, G. Lambrou,

V.C. Lansingh, P. Lanzetta, J. Lim, K. Mansouri, A. Mathew, A. Morse, D. Musch, V. Nangia, M. Battaglia, F. Yaacov, M. Raju, L. Rossetti, J. Saaddine, M. Sandar, J. Serle, R. Shetty, P. Sieving, J.C. Silva, R.S. Sitorus, J. Tejedor, M. Tsilimbaris, J. van Meurs, R. Varma, G. Virgili, J. Volmink, Y. Xing, N.L. Wang, P. Wiedemann, Y. Zheng, Magnitude, temporal trends, and projections of the global prevalence of blindness and distance and near vision impairment: a systematic review and meta-analysis, Lancet Glob. Heal. 5 (2017) e888–e897. doi:10.1016/S2214-109X(17)30293-0.

[35] A. Bastawrous, A.-V. Suni, Thirty Year Projected Magnitude (to 2050) of Near and Distance Vision Impairment and the Economic Impact if Existing Solutions are Implemented Globally, Ophthalmic Epidemiol. 27 (2020) 115–120. doi:10.1080/09286586.2019.1700532.

[36] World report on vision, (2019). https://www.who.int/publications/i/item/world-report-on-vision (accessed November 3, 2020).

[37] R.P. Danis, J.A. Lavine, A. Domalpally, Geographic atrophy in patients with advanced dry age-related macular degeneration: Current challenges and future prospects, Clin. Ophthalmol. 9 (2015) 2159–2174. doi:10.2147/OPTH.S92359.

[38] W.L. Wong, X. Su, X. Li, C.M.G. Cheung, R. Klein, C.Y. Cheng, T.Y. Wong, Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: A systematic review and meta-analysis, Lancet Glob. Heal. 2 (2014). doi:10.1016/S2214-109X(13)70145-1.

[39] J. Zhang, J. Tuo, Z. Wang, A. Zhu, A. Machalińska, Q. Long, Pathogenesis of common ocular diseases, J. Ophthalmol. 2015 (2015). doi:10.1155/2015/734527.

[40] J.T. Handa, C. Bowes Rickman, A.D. Dick, M.B. Gorin, J.W. Miller, C.A. Toth, M. Ueffing, M. Zarbin, L.A. Farrer, A systems biology approach towards understanding and treating non-neovascular age-related macular degeneration, Nat. Commun. 10 (2019). doi:10.1038/s41467-019-11262-1.

[41] L. V. Johnson, S. Ozaki, M.K. Staples, P.A. Erickson, D.H. Anderson, A potential role for immune complex pathogenesis in drusen formation, Exp. Eye Res. 70 (2000) 441–449. doi:10.1006/exer.1999.0798.

[42] O. Arjamaa, M. Nikinmaa, A. Salminen, K. Kaarniranta, Regulatory role of HIF-1α in the pathogenesis of age-related macular degeneration (AMD), Ageing Res. Rev. 8 (2009) 349–358. doi:10.1016/j.arr.2009.06.002.

[43] M. Singer, Advances in the management of macular degeneration, F1000Prime Rep. 6 (2014). doi:10.12703/P6-29.

[44] N. Ferrara, VEGF and Intraocular Neovascularization: From Discovery to Therapy, Sci Tech. 5 (2016) 10. doi:10.1167/tvst.5.2.10.

[45] Y. Oshima, S. Oshima, H. Nambu, S. Kachi, S.F. Hackett, M. Melia, M. Kaleko, S. Connelly, N. Esumi, D.J. Zack, P.A. Campochiaro, Increased expression of VEGF in retinal pigmented epithelial cells is not sufficient to cause choroidal neovascularization, J. Cell. Physiol. 201 (2004) 393–400. doi:10.1002/jcp.20110.

[46] R. Tuuminen, H. Uusitalo-Järvinen, V. Aaltonen, N. Hautala, S. Kaipiainen, N. Laitamäki, M. Ollila, J. Rantanen, S. Välimäki, R. Sipilä, T. Laukkala, J.

Komulainen, P. Tommila, I. Immonen, A. Tuulonen, K. Kaarniranta, The Finnish national guideline for diagnosis, treatment and follow-up of patients with wet age-related macular degeneration, Acta Ophthalmol. 95 (2017) 1–9. doi:10.1111/aos.13501.

[47] X. Cai, J.F. McGinnis, Diabetic Retinopathy: Animal Models, Therapies, and Perspectives, J. Diabetes Res. 2016 (2016). doi:10.1155/2016/3789217.

[48] N. Cheung, P. Mitchell, T.Y. Wong, Diabetic retinopathy, in: Lancet, Elsevier Limited, 2010: pp. 124–136. doi:10.1016/S0140-6736(09)62124-3.

[49] I. Klaassen, C.J.F. Van Noorden, R.O. Schlingemann, Molecular basis of the inner blood-retinal barrier and its breakdown in diabetic macular edema and other pathological conditions, Prog. Retin. Eye Res. 34 (2013) 19–48. doi:10.1016/j.preteyeres.2013.02.001.

[50] P.A. Campochiaro, Retinal and choroidal neovascularization, J. Cell. Physiol. 184 (2000) 301–310. doi:10.1002/1097-4652(200009)184:3<301::AID-JCP3>3.0.CO;2-H.

[51] J. Zhang, J. Tuo, Z. Wang, A. Zhu, A. Machalinska, Q. Long, Editorial Pathogenesis of Common Ocular Diseases, (2015). doi:10.1155/2015/734527.

[52] J. Qu, D. Wang, C.L. Grosskreutz, Mechanisms of retinal ganglion cell injury and defense in glaucoma, Exp. Eye Res. 91 (2010) 48–53. doi:10.1016/j.exer.2010.04.002.

[53] S. Alqawlaq, J.T. Huzil, M. V. Ivanova, M. Foldvari, Challenges in neuroprotective nanomedicine development: Progress towards noninvasive gene therapy of glaucoma, Nanomedicine. 7 (2012) 1067–1083. doi:10.2217/nnm.12.69.

[54] K. Trier, The Biology of the Eye, Volume 10 - 1st Edition, Elsevier. (2006) 353–373. https://www.elsevier.com/books/the-biology-of-the-eye/fischbarg/978-0-444-50925-3 (accessed November 18, 2020).

[55] V. Gote, S. Sikder, J. Sicotte, D. Pal, Special Section on Drug Delivery Technologies-Minireview Ocular Drug Delivery: Present Innovations and Future Challenges, J. Pharmacol. Exp. Ther. J Pharmacol Exp Ther. 370 (2019) 602–624. doi:10.1124/jpet.119.256933.

[56] I. Pal Kaur, M. Kanwar, Ocular Preparations: The Formulation Approach, Drug Dev. Ind. Pharm. 28 (2002) 473–493. doi:10.1081/DDC-120003445.

[57] A. Subrizi, E.M. del Amo, V. Korzhikov-Vlakh, T. Tennikova, M. Ruponen, A. Urtti, Design principles of ocular drug delivery systems: importance of drug payload, release rate, and material properties, Drug Discov. Today. 24 (2019) 1446–1457. doi:10.1016/j.drudis.2019.02.001.

[58] A. Fayyaz, V.P. Ranta, E. Toropainen, K.S. Vellonen, A. Valtari, J. Puranen, M. Ruponen, I. Gardner, A. Urtti, M. Jamei, E.M. del Amo, Topical ocular pharmacokinetics and bioavailability for a cocktail of atenolol, timolol and betaxolol in rabbits, Eur. J. Pharm. Sci. 155 (2020) 105553. doi:10.1016/j.ejps.2020.105553.

[59] V. Naageshwaran, V.P. Ranta, G. Gum, S. Bhoopathy, A. Urtti, E.M. del Amo, Comprehensive Ocular and Systemic Pharmacokinetics of Brinzolamide in Rabbits

After Intracameral, Topical, and Intravenous Administration, J. Pharm. Sci. 110 (2021) 529–535. doi:10.1016/j.xphs.2020.09.051.

[60] D.M. Maurice, S. Mishima, Ocular Pharmacokinetics, in: Pharmacol. Eye. Handb. Exp. Pharmacol. Springer, Springer, Berlin, Heidelberg, 1984: pp. 19–116. doi:10.1007/978-3-642-69222-2_2.

[61] W. Zhang, M.R. Prausnitz, A. Edwards, Model of transient drug diffusion across cornea, J. Control. Release. 99 (2004) 241–258. doi:10.1016/j.jconrel.2004.07.001.

[62] R.D. Schoenwald, The Importance of Optimizing Corneal Penetration, in: Ophthalmic Drug Deliv., Springer New York, 1987: pp. 151–160. doi:10.1007/978-1-4757-4175-9_15.

[63] R.D. Schoenwald, Ocular Drug Delivery: Pharmacokinetic Considerations, Clin. Pharmacokinet. 18 (1990) 255–269. doi:10.2165/00003088-199018040-00001.

[64] M. Hornof, E. Toropainen, A. Urtti, Cell culture models of the ocular barriers, in: Eur. J. Pharm. Biopharm., Elsevier, 2005: pp. 207–225. doi:10.1016/j.ejpb.2005.01.009.

[65] D.W. DelMonte, T. Kim, Anatomy and physiology of the cornea, J. Cataract Refract. Surg. 37 (2011) 588–598. doi:10.1016/j.jcrs.2010.12.037.

[66] H. Kidron, K.S. Vellonen, E.M. Del Amo, A. Tissari, A. Urtti, Prediction of the corneal permeability of drug-like compounds, Pharm. Res. 27 (2010) 1398–1407. doi:10.1007/s11095-010-0132-8.

[67] A.K. Mitra, Treatise on Ocular Drug Delivery - Google Books, Bentham Sci. Publ. (2013). https://books.google.fi/books/about/Treatise_on_Ocular_Drug_Delivery.html?id=LkriAwAAQBAJ&redir_esc=y (accessed November 20, 2020).

[68] A.T.F. Patton, Importance of the noncorneal absorption route in topical ophthalmic drug delivery. | IOVS | ARVO Journals, IOVS. (1985). https://iovs.arvojournals.org/article.aspx?articleid=2177115 (accessed December 10, 2020).

[69] E. Ramsay, E.M. del Amo, E. Toropainen, U. Tengvall-Unadike, V.P. Ranta, A. Urtti, M. Ruponen, Corneal and conjunctival drug permeability: Systematic comparison and pharmacokinetic impact in the eye, Eur. J. Pharm. Sci. 119 (2018) 83–89. doi:10.1016/j.ejps.2018.03.034.

[70] S. H.S. Boddu, H. Gupta, S. Patel, Drug Delivery to the Back of the Eye Following Topical Administration: An Update on Research and Patenting Activity, Recent Pat. Drug Deliv. Formul. 8 (2014) 27–36.

[71] G.A. Rodrigues, D. Lutz, J. Shen, X. Yuan, H. Shen, J. Cunningham, H.M. Rivers, Topical Drug Delivery to the Posterior Segment of the Eye: Addressing the Challenge of Preclinical to Clinical Translation, Pharm. Res. 35 (2018). doi:10.1007/s11095-018-2519-x.

[72] R. Vadlapatla, A. Vadlapudi, D. Pal, A. Mitra, Role of Membrane Transporters and Metabolizing Enzymes in Ocular Drug Delivery, Curr. Drug Metab. 15 (2015) 680–693. doi:10.2174/1389200215666140926152459.

[73] K.S. Vellonen, E.M. Soini, E.M. Del Amo, A. Urtti, Prediction of ocular drug distribution from systemic blood circulation, Mol. Pharm. 13 (2016) 2906–2911. doi:10.1021/acs.molpharmaceut.5b00729.

[74] L. Pitkänen, V.P. Ranta, H. Moilanen, A. Urtti, Permeability of retinal pigment epithelium: Effects of permeant molecular weight and lipophilicity, Investig. Ophthalmol. Vis. Sci. 46 (2005) 641–646. doi:10.1167/iovs.04-1051.

[75] D. Maurice, Review: Practical issues in intravitreal drug delivery, J. Ocul. Pharmacol. Ther. 17 (2001) 393–401. doi:10.1089/108076801753162807.

[76] K. Peynshaert, J. Devoldere, S.C. De Smedt, K. Remaut, In vitro and ex vivo models to study drug delivery barriers in the posterior segment of the eye, Adv. Drug Deliv. Rev. 126 (2018) 44–57. doi:10.1016/j.addr.2017.09.007.

[77] L. Pitkänen, M. Ruponen, J. Nieminen, A. Urtti, Vitreous is a barrier in nonviral gene transfer by cationic lipids and polymers, Pharm. Res. 20 (2003) 576–583. doi:10.1023/A:1023238530504.

[78] S. Tavakoli, O.K. Kari, T. Turunen, T. Lajunen, M. Schmitt, J. Lehtinen, F. Tasaka, P. Parkkila, J. Ndika, T. Viitala, H. Alenius, A. Urtti, A. Subrizi, Diffusion and Protein Corona Formation of Lipid-Based Nanoparticles in the Vitreous Humor: Profiling and Pharmacokinetic Considerations, Mol. Pharm. (2020). doi:10.1021/acs.molpharmaceut.0c00411.

[79] P.N. Bishop, D.F. Holmes, K.E. Kadler, D. McLeod, K.J. Bos, Age-related changes on the surface of vitreous collagen fibrils., Invest. Ophthalmol. Vis. Sci. 45 (2004) 1041–6. doi:10.1167/iovs.03-1017.

[80] E.M. Del Amo, K.S. Vellonen, H. Kidron, A. Urtti, Intravitreal clearance and volume of distribution of compounds in rabbits: In silico prediction and pharmacokinetic simulations for drug development, Eur. J. Pharm. Biopharm. 95 (2015) 215–226. doi:10.1016/j.ejpb.2015.01.003.

[81] H. Kidron, E.M. Del Amo, K.S. Vellonen, A. Urtti, Prediction of the vitreal half-life of small molecular drug-like compounds, Pharm. Res. 29 (2012) 3302–3311. doi:10.1007/s11095-012-0822-5.

[82] H. Fuchs, F. Igney, Binding to Ocular Albumin as a Half-Life Extension Principle for Intravitreally Injected Drugs: Evidence from Mechanistic Rat and Rabbit Studies, J. Ocul. Pharmacol. Ther. 33 (2017) 115–122. doi:10.1089/jop.2016.0083.

[83] L.A. Hutton-Smith, E.A. Gaffney, H.M. Byrne, A. Caruso, P.K. Maini, N.A. Mazer, Theoretical Insights into the Retinal Dynamics of Vascular Endothelial Growth Factor in Patients Treated with Ranibizumab, Based on an Ocular Pharmacokinetic/Pharmacodynamic Model, Mol. Pharm. 15 (2018) 2770–2784. doi:10.1021/acs.molpharmaceut.8b00280.

[84] S. Tavakoli, K. Peynshaert, T. Lajunen, J. Devoldere, E.M. del Amo, M. Ruponen, S.C. De Smedt, K. Remaut, A. Urtti, Ocular barriers to retinal delivery of intravitreal liposomes: Impact of vitreoretinal interface, J. Control. Release. (2020). doi:10.1016/j.jconrel.2020.10.028.

[85] L.A. Hutton-Smith, E.A. Gaffney, H.M. Byrne, P.K. Maini, K. Gadkar, N.A. Mazer, Ocular Pharmacokinetics of Therapeutic Antibodies Given by Intravitreal Injection:

Estimation of Retinal Permeabilities Using a 3-Compartment Semi-Mechanistic Model, Mol. Pharm. 14 (2017) 2690–2696. doi:10.1021/acs.molpharmaceut.7b00164.

[86] J. Candiello, G.J. Cole, W. Halfter, Age-dependent changes in the structure, composition and biophysical properties of a human basement membrane, Matrix Biol. 29 (2010) 402–410. doi:10.1016/j.matbio.2010.03.004.

[87] W. Halfter, P. Oertle, C.A. Monnier, L. Camenzind, M. Reyes-Lua, H. Hu, J. Candiello, A. Labilloy, M. Balasubramani, P.B. Henrich, M. Plodinec, New concepts in basement membrane biology, FEBS J. 282 (2015) 4466–4479. doi:10.1111/febs.13495.

[88] F. Malecaze, C. Caratero, A. Caratero, J.L. Arne, A. Mathis, P. Bec, H. Planel, Some Ultrastructural Aspects of the Vitreoretinal Juncture, Ophthalmologica. 191 (1985) 22–28. doi:10.1159/000309534.

[89] R.Y. Foos, Vitreoretinal juncture; topographical variations - PubMed, Invest Ophthalmol Vis Sci. (1972) 801–808. https://pubmed.ncbi.nlm.nih.gov/4561129/ (accessed December 15, 2020).

[90] J. Sebag, Anatomy and pathology of the vitreo-retinal interface, Eye. 6 (1992) 541–552. doi:10.1038/eye.1992.119.

[91] S. Heegaard, Structure of the Human Vitreoretinal Border Region, Ophthalmologica. 208 (1994) 82–91. doi:10.1159/000310458.

[92] T.L. Ponsioen, M.J.A. van Luyn, R.J. van der Worp, H.H. Pas, J.M.M. Hooymans, L.I. Los, Human retinal Müller cells synthesize collagens of the vitreous and vitreoretinal interface in vitro, Mol. Vis. 14 (2008) 652–660.

[93] S. Roy, M. Maiello, M. Lorenzi, Increased expression of basement membrane collagen in human diabetic retinopathy., J. Clin. Invest. 93 (1994) 438. doi:10.1172/JCI116979.

[94] K.D. Kolstad, D. Dalkara, K. Guerin, M. Visel, N. Hoffmann, D. V. Schaffer, J.G. Flannery, Changes in adeno-associated virus-mediated gene delivery in retinal degeneration, Hum. Gene Ther. 21 (2010) 571–578. doi:10.1089/hum.2009.194.

[95] O. Vacca, M. Darche, D. V. Schaffer, J.G. Flannery, J.A. Sahel, A. Rendon, D. Dalkara, AAV-mediated gene delivery in Dp71-null mouse model with compromised barriers, Glia. 62 (2014) 468–476. doi:10.1002/glia.22617.

[96] W. Halfter, J. Sebag, E.T. Cunningham, Vitreoretinal interface and inner limiting membrane, in: Vitr. Heal. Dis., Springer New York, 2014: pp. 165–191. doi:10.1007/978-1-4939-1086-1_11.

[97] L. Pitkänen, J. Pelkonen, M. Ruponen, S. Rönkkö, A. Urtti, Neural retina limits the nonviral gene transfer to retinal pigment epithelium in an in vitro bovine eye model, AAPS J. 6 (2004) 72–80. doi:10.1208/aapsj060325.

[98] J. GAUDREAULT, D. FEI, J.C. BEYER, A. RYAN, L. RANGELL, V. SHIU, L.A. DAMICO, PHARMACOKINETICS AND RETINAL DISTRIBUTION OF RANIBIZUMAB, A HUMANIZED ANTIBODY FRAGMENT DIRECTED AGAINST VEGF-A, FOLLOWING INTRAVITREAL ADMINISTRATION IN

RABBITS, Retina. 27 (2007) 1260–1266. doi:10.1097/IAE.0b013e318134eecd.

[99] P. Heiduschka, H. Fietz, S. Hofmeister, S. Schultheiss, A.F. Mack, S. Peters, F. Ziemssen, B. Niggemann, S. Julien, K.U. Bartz-Schmidt, U. Schraermeyer, Penetration of bevacizumab through the retina after intravitreal injection in the monkey, Investig. Ophthalmol. Vis. Sci. 48 (2007) 2814–2823. doi:10.1167/iovs.06-1171.

[100] J.S. PULIDO, S.J. BAKRI, T. VALYI-NAGY, D. SHUKLA, RITUXIMAB PENETRATES FULL-THICKNESS RETINA IN CONTRAST TO TISSUE PLASMINOGEN ACTIVATOR CONTROL, Retina. 27 (2007) 1071–1073. doi:10.1097/IAE.0b013e31804ac032.

[101] J. Mordenti, R.A. Cuthbertson, N. Ferrara, K. Thomsen, L. Berleau, V. Licko, P.C. Allen, C.R. Valverde, Y.G. Meng, D.T. w. Fei, K.M. Fourre, A.M. Ryan, Comparisons of the Intraocular Tissue Distribution, Pharmacokinetics, and Safety of 125I-Labeled Full-Length and Fab Antibodies in Rhesus Monkeys Following Intravitreal Administration, Toxicol. Pathol. 27 (1999) 536–544. doi:10.1177/019262339902700507.

[102] R. Gaudana, J. Jwala, S.H.S. Boddu, A.K. Mitra, Recent perspectives in ocular drug delivery, Pharm. Res. 26 (2009) 1197–1216. doi:10.1007/s11095-008-9694-0.

[103] C. Durairaj, Ocular pharmacokinetics, in: Handb. Exp. Pharmacol., Springer New York LLC, 2017: pp. 31–55. doi:10.1007/164_2016_32.

[104] H. Kim, M.R. Robinson, M.J. Lizak, G. Tansey, R.J. Lutz, P. Yuan, N.S. Wang, K.G. Csaky, Controlled drug release from an ocular implant: An evaluation using dynamic three-dimensional magnetic resonance imaging, Investig. Ophthalmol. Vis. Sci. 45 (2004) 2722–2731. doi:10.1167/iovs.04-0091.

[105] A.C. Amrite, H.F. Edelhauser, S.R. Singh, U.B. Kompella, Effect of circulation on the disposition and ocular tissue distribution of 20 nm nanoparticles after periocular administration, Mol. Vis. 14 (2008) 150–160. http://www.molvis.org/molvis/v14/a20 (accessed December 17, 2020).

[106] V.P. Ranta, E. Mannermaa, K. Lummepuro, A. Subrizi, A. Laukkanen, M. Antopolsky, L. Murtomäki, M. Hornof, A. Urtti, Barrier analysis of periocular drug delivery to the posterior segment, J. Control. Release. 148 (2010) 42–48. doi:10.1016/j.jconrel.2010.08.028.

[107] D. Ghate, W. Brooks, B.E. McCarey, H.F. Edelhauser, Pharmacokinetics of intraocular drug delivery by periocular injections using ocular fluorophotometry, Investig. Ophthalmol. Vis. Sci. 48 (2007) 2230–2237. doi:10.1167/iovs.06-0954.

[108] P. Tyagi, R.S. Kadam, U.B. Kompella, Comparison of Suprachoroidal Drug Delivery with Subconjunctival and Intravitreal Routes Using Noninvasive Fluorophotometry, PLoS One. 7 (2012) e48188. doi:10.1371/journal.pone.0048188.

[109] A.S. Willoughby, V.S. Vuong, D. Cunefare, S. Farsiu, G. Noronha, R.P. Danis, G. Yiu, Choroidal Changes After Suprachoroidal Injection of Triamcinolone Acetonide in Eyes With Macular Edema Secondary to Retinal Vein Occlusion, Am. J. Ophthalmol. 186 (2018) 144–151. doi:10.1016/j.ajo.2017.11.020.

[110] Clearside BIO : Programs, (n.d.). http://www.clearsidebio.com/programs.htm

(accessed December 17, 2020).

[111] I. Pascual-Camps, P. Hernández-Martínez, L. Monje-Fernández, R. Dolz-Marco, R. Gallego-Pinazo, L. Wu, J.F. Arévalo, M. Díaz-Llopis, Update on intravitreal anti-tumor necrosis factor alpha therapies for ocular disorders, J. Ophthalmic Inflamm. Infect. 4 (2014) 1–5. doi:10.1186/s12348-014-0026-8.

[112] K. Nayak, M. Misra, A review on recent drug delivery systems for posterior segment of eye, Biomed. Pharmacother. 107 (2018) 1564–1582. doi:10.1016/j.biopha.2018.08.138.

[113] T. Yasukawa, Y. Tabata, H. Kimura, Y. Ogura, Recent Advances in Intraocular Drug Delivery Systems, Recent Pat. Drug Deliv. Formul. 5 (2011) 1–10. doi:10.2174/187221111794109529.

[114] J.F. Fangueiro, A.M. Silva, M.L. Garcia, E.B. Souto, Current nanotechnology approaches for the treatment and management of diabetic retinopathy, Eur. J. Pharm. Biopharm. 95 (2015) 307–322. doi:10.1016/j.ejpb.2014.12.023.

[115] P.U. Dugel, A. Koh, Y. Ogura, G.J. Jaffe, U. Schmidt-Erfurth, D.M. Brown, A. V. Gomes, J. Warburton, A. Weichselberger, F.G. Holz, HAWK and HARRIER: Phase 3, Multicenter, Randomized, Double-Masked Trials of Brolucizumab for Neovascular Age-Related Macular Degeneration, in: Ophthalmology, Elsevier Inc., 2020: pp. 72–84. doi:10.1016/j.ophtha.2019.04.017.

[116] Beovu (brolucizumab intravitreal) dosing, indications, interactions, adverse effects, and more, (n.d.). https://reference.medscape.com/drug/beovu-brolucizumab-intravitreal-1000350#10 (accessed December 2, 2020).

[117] R.M. Hussain, C.Y. Weng, C.C. Wykoff, R.A. Gandhi, S.M. Hariprasad, Abicipar pegol for neovascular age-related macular degeneration, Expert Opin. Biol. Ther. 20 (2020) 1–9. doi:10.1080/14712598.2020.1782379.

[118] J.K. Patel, V. Sutariya, J.R. Kanwar, Y. V. Pathak, Drug delivery for the retina and posterior segment disease, Springer International Publishing, 2018. doi:10.1007/978-3-319-95807-1.

[119] M.W. Stewart, Treatment of diabetic retinopathy: Recent advances and unresolved challenges, World J. Diabetes. 7 (2016) 333. doi:10.4239/wjd.v7.i16.333.

[120] P.J. Rosenfeld, D.M. Brown, J.S. Heier, D.S. Boyer, P.K. Kaiser, C.Y. Chung, R.Y. Kim, Ranibizumab for Neovascular Age-Related Macular Degeneration, N. Engl. J. Med. 355 (2006) 1419–1431. doi:10.1056/nejmoa054481.

[121] B.G. Busbee, A.C. Ho, D.M. Brown, J.S. Heier, I.J. Suñer, Z. Li, R.G. Rubio, P. Lai, Twelve-month efficacy and safety of 0.5 mg or 2.0 mg ranibizumab in patients with subfoveal neovascular age-related macular degeneration, Ophthalmology. 120 (2013) 1046–1056. doi:10.1016/j.ophtha.2012.10.014.

[122] K.G. Janoria, S. Gunda, S.H.S. Boddu, A.K. Mitra, Novel approaches to retinal drug delivery, Expert Opin. Drug Deliv. 4 (2007) 371–388. doi:10.1517/17425247.4.4.371.

[123] G.A. Peyman, E.M. Lad, D.M. Moshfeghi, Intravitreal injection of therapeutic agents, Retina. 29 (2009) 875–912. doi:10.1097/IAE.0b013e3181a94f01.

[124] H. Wu, T.C. Chen, The effects of intravitreal ophthalmic medications on intraocular pressure, Semin. Ophthalmol. 24 (2009) 100–105. doi:10.1080/08820530902800397.

[125] R. Lazic, M. Lukic, I. Boras, N. Draca, M. Vlasic, N. Gabric, Z. Tomic, Treatment of anti-vascular endothelial growth factor-resistant diabetic macular edema with dexamethasone intravitreal implant, Retina. 34 (2014) 719–724. doi:10.1097/IAE.0b013e3182a48958.

[126] S.A. Gaballa, U.B. Kompella, O. Elgarhy, A.M. Alqahtani, B. Pierscionek, R.G. Alany, H. Abdelkader, Corticosteroids in ophthalmology: drug delivery innovations, pharmacology, clinical applications, and future perspectives, Drug Deliv. Transl. Res. (2020). doi:10.1007/s13346-020-00843-z.

[127] L.C. Zacharias, T. Lin, R. Migon, C. Ghosn, W. Orilla, B. Feldmann, G. Ruiz, Y. Li, J. Burke, B.D. Kuppermann, ASSESSMENT OF THE DIFFERENCES IN PHARMACOKINETICS AND PHARMACODYNAMICS BETWEEN FOUR DISTINCT FORMULATIONS OF TRIAMCINOLONE ACETONIDE, Retina. 33 (2013) 522–531. doi:10.1097/IAE.0b013e3182647f69.

[128] U.B. Kompella, R.S. Kadam, V.H.L. Lee, Recent advances in ophthalmic drug delivery, Ther. Deliv. 1 (2010) 435–456. doi:10.4155/tde.10.40.

[129] V. Delplace, S. Payne, M. Shoichet, Delivery strategies for treatment of age-related ocular diseases: From a biological understanding to biomaterial solutions, J. Control. Release. 219 (2015) 652–668. doi:10.1016/j.jconrel.2015.09.065.

[130] S.J. Bakri, A.F. Omar, Evolution of vitreomacular traction following the use of the dexamethasone intravitreal implant (ozurdex) in the treatment of macular edema secondary to central retinal vein occlusion, J. Ocul. Pharmacol. Ther. 28 (2012) 547–549. doi:10.1089/jop.2011.0184.

[131] J.B. Rice, A.G. White, L.M. Scarpati, G. Wan, W.W. Nelson, Long-term Systemic Corticosteroid Exposure: A Systematic Literature Review, Clin. Ther. 39 (2017) 2216–2229. doi:10.1016/j.clinthera.2017.09.011.

[132] D. Yadav, L.T. Varma, K. Yadav, Drug delivery to posterior segment of the eye: Conventional delivery strategies, their barriers, and restrictions, in: Drug Deliv. Retin. Posterior Segm. Dis., Springer International Publishing, 2018: pp. 51–67. doi:10.1007/978-3-319-95807-1_3.

[133] S. Iyer, A.E. Radwan, A. Hafezi-Moghadam, P. Malyala, M. Amiji, Long-acting intraocular Delivery strategies for biological therapy of age-related macular degeneration, J. Control. Release. 296 (2019) 140–149. doi:10.1016/j.jconrel.2019.01.007.

[134] F. Ramazani, C. Hiemstra, R. Steendam, F. Kazazi-Hyseni, C.F. Van Nostrum, G. Storm, F. Kiessling, T. Lammers, W.E. Hennink, R.J. Kok, Sunitinib microspheres based on [PDLLA-PEG-PDLLA]-b-PLLA multi-block copolymers for ocular drug delivery, Eur. J. Pharm. Biopharm. 95 (2015) 368–377. doi:10.1016/j.ejpb.2015.02.011.

[135] A. Giddabasappa, K. Lalwani, R. Norberg, H.J. Gukasyan, D. Paterson, R.A. Schachar, K. Rittenhouse, K. Klamerus, L. Mosyak, J. Eswaraka, Axitinib inhibits

retinal and choroidal neovascularization in in vitro and in vivo models, Exp. Eye Res. 145 (2016) 373–379. doi:10.1016/j.exer.2016.02.010.

[136] C.A. Puliafito, C.C. Wykoff, Looking ahead in retinal disease management: highlights of the 2019 angiogenesis, exudation and degeneration symposium, Int. J. Retin. Vitr. 5 (2019). doi:10.1186/s40942-019-0174-y.

[137] D.S. Welsbie, K.L. Mitchell, V. Jaskula-Ranga, V.M. Sluch, Z. Yang, J. Kim, E. Buehler, A. Patel, S.E. Martin, P.-W. Zhang, Y. Ge, Y. Duan, J. Fuller, B.-J. Kim, E. Hamed, X. Chamling, L. Lei, I.D. Fraser, Z.A. Ronai, C.A. Berlinicke, D.J. Zack, Enhanced Functional Genomic Screening Identifies Novel Mediators of Dual Leucine Zipper Kinase-Dependent Injury Signaling in Neurons, (2017). doi:10.1016/j.neuron.2017.06.008.

[138] D.S. Welsbie, N.K. Ziogas, L. Xu, B.J. Kim, Y. Ge, A.K. Patel, J. Ryu, M. Lehar, A.S. Alexandris, N. Stewart, D.J. Zack, V.E. Koliatsos, Targeted disruption of dual leucine zipper kinase and leucine zipper kinase promotes neuronal survival in a model of diffuse traumatic brain injury, Mol. Neurodegener. 14 (2019) 44. doi:10.1186/s13024-019-0345-1.

[139] Pipeline – Ocular Therapeutix, (n.d.). https://www.ocutx.com/research/pipeline/ (accessed December 8, 2020).

[140] H. Al-Khersan, R.M. Hussain, T.A. Ciulla, P.U. Dugel, Innovative therapies for neovascular age-related macular degeneration, Expert Opin. Pharmacother. 20 (2019) 1879–1891. doi:10.1080/14656566.2019.1636031.

[141] Pipeline - EyePoint Pharmaceuticals, Inc., (n.d.). https://eyepointpharma.com/pipeline/ (accessed December 8, 2020).

[142] S.J. Robbie, P.L. von Leithner, M. Ju, C.A. Lange, A.G. King, P. Adamson, D. Lee, C. Sychterz, P. Coffey, Y.S. Ng, J.W. Bainbridge, D.T. Shima, Assessing a novel depot delivery strategy for noninvasive administration of VEGF/PDGF RTK inhibitors for ocular neovascular disease, Investig. Ophthalmol. Vis. Sci. 54 (2013) 1490–1500. doi:10.1167/iovs.12-10169.

[143] J. Kim, T.E. Kim, J.A. Kim, J.H. Yun, S. Sohn, S.R. Shim, S.H. Lee, S.J. Kim, Intravitreal tanibirumab, a fully human monoclonal antibody against vascular endothelial growth factor receptor 2, partially suppresses and regresses laser-induced choroidal neovascularization in a rat model, J. Ocul. Pharmacol. Ther. 30 (2014) 847–853. doi:10.1089/jop.2014.0021.

[144] N. Jo, C. Mailhos, M. Ju, E. Cheung, J. Bradley, K. Nishijima, G.S. Robinson, A.P. Adamis, D.T. Shima, Inhibition of platelet-derived growth factor B signaling enhances the efficacy of anti-vascular endothelial growth factor therapy in multiple models of ocular neovascularization, Am. J. Pathol. 168 (2006) 2036–2053. doi:10.2353/ajpath.2006.050588.

[145] R.D. Jager, W.F. Mieler, J.W. Miller, Age-Related Macular Degeneration, N. Engl. J. Med. 358 (2008) 2606–2617. doi:10.1056/NEJMra0801537.

[146] B.J. Kim, D.C. Mastellos, Y. Li, J.L. Dunaief, J.D. Lambris, Targeting complement components C3 and C5 for the retina: Key concepts and lingering questions, Prog. Retin. Eye Res. (2020) 100936. doi:10.1016/j.preteyeres.2020.100936.

[147] S. Iyer, A.E. Radwan, A. Hafezi-Moghadam, P. Malyala, M. Amiji, Long-acting intraocular Delivery strategies for biological therapy of age-related macular degeneration, J. Control. Release. 296 (2019) 140–149. doi:10.1016/j.jconrel.2019.01.007.

[148] H. Xu, M. Chen, Targeting the complement system for the management of retinal inflammatory and degenerative diseases, Eur. J. Pharmacol. 787 (2016) 94–104. doi:10.1016/j.ejphar.2016.03.001.

[149] W. Tao, R. Wen, M.B. Goddard, S.D. Sherman, P.J. O'Rourke, P.F. Stabila, W.J. Bell, B.J. Dean, K.A. Kauper, V.A. Budz, W.G. Tsiaras, G.M. Acland, S. Pearce-Kelling, A.M. Laties, G.D. Aguirre, Encapsulated cell-based delivery of CNTF reduces photoreceptor degeneration in animal models of retinitis pigmentosa, Investig. Ophthalmol. Vis. Sci. 43 (2002) 3292–3298. http://intl.iovs.org/cgi/content/full/43/10/3292 (accessed December 7, 2020).

[150] V. Gote, S. Sikder, J. Sicotte, D. Pal, Ocular drug delivery: Present innovations and future challenges, J. Pharmacol. Exp. Ther. 370 (2019) 602–624. doi:10.1124/jpet.119.256933.

[151] Clinical Trial on Glaucoma: NT-501, Sham comparator - Clinical Trials Registry - ICH GCP, (2020). https://ichgcp.net/clinical-trials-registry/NCT04577300 (accessed December 7, 2020).

[152] J. Jiang, X. Zhang, Y. Tang, S. Li, J. Chen, Progress on ocular siRNA gene-silencing therapy and drug delivery systems, Fundam. Clin. Pharmacol. (2020) fcp.12561. doi:10.1111/fcp.12561.

[153] E. Fattal, A. Bochot, State of the art and perspectives for the delivery of antisense oligonucleotides and siRNA by polymeric nanocarriers, Int. J. Pharm. 364 (2008) 237–248. doi:10.1016/j.ijpharm.2008.06.011.

[154] S. Naik, A.B. Shreya, R. Raychaudhuri, A. Pandey, S.A. Lewis, M. Hazarika, S. V. Bhandary, B.S.S. Rao, S. Mutalik, Small interfering RNAs (siRNAs) based gene silencing strategies for the treatment of glaucoma: Recent advancements and future perspectives, Life Sci. 264 (2021) 118712. doi:10.1016/j.lfs.2020.118712.

[155] I. Trapani, A. Auricchio, Seeing the Light after 25 Years of Retinal Gene Therapy, Trends Mol. Med. 24 (2018) 669–681. doi:10.1016/j.molmed.2018.06.006.

[156] E. Fattal, A. Bochot, Ocular delivery of nucleic acids: antisense oligonucleotides, aptamers and siRNA, Adv. Drug Deliv. Rev. 58 (2006) 1203–1223. doi:10.1016/j.addr.2006.07.020.

[157] A. Wittrup, J. Lieberman, Knocking down disease: A progress report on siRNA therapeutics, Nat. Rev. Genet. 16 (2015) 543–552. doi:10.1038/nrg3978.

[158] A. Auricchio, A.J. Smith, R.R. Ali, The Future Looks Brighter After 25 Years of Retinal Gene Therapy, Hum. Gene Ther. 28 (2017) 982–987. doi:10.1089/hum.2017.164.

[159] C. Schön, M. Biel, S. Michalakis, Retinal gene delivery by adeno-associated virus (AAV) vectors: Strategies and applications, Eur. J. Pharm. Biopharm. 95 (2015) 343–352. doi:10.1016/j.ejpb.2015.01.009.

[160] ADVM-022 Intravitreal Gene Therapy for Wet AMD - Full Text View - ClinicalTrials.gov, (n.d.). https://clinicaltrials.gov/ct2/show/NCT03748784 (accessed January 29, 2021).

[161] P.A. Campochiaro, D.M. Marcus, C.C. Awh, C. Regillo, A.P. Adamis, V. Bantseev, Y. Chiang, J.S. Ehrlich, S. Erickson, W.D. Hanley, J. Horvath, K.F. Maass, N. Singh, F. Tang, G. Barteselli, The Port Delivery System with Ranibizumab for Neovascular Age-Related Macular Degeneration: Results from the Randomized Phase 2 Ladder Clinical Trial, Ophthalmology. 126 (2019) 1141–1154. doi:10.1016/j.ophtha.2019.03.036.

[162] C.A. Puliafito, C.C. Wykoff, New Frontiers in Retina: Highlights of the 2020 angiogenesis, exudation and degeneration symposium, Int. J. Retin. Vitr. 6 (2020) 18. doi:10.1186/s40942-020-00221-4.

[163] I. Seah, X. Zhao, Q. Lin, Z. Liu, S.Z.Z. Su, Y. Sen Yuen, W. Hunziker, G. Lingam, X.J. Loh, X. Su, Use of biomaterials for sustained delivery of anti-VEGF to treat retinal diseases, Eye. 34 (2020) 1341–1356. doi:10.1038/s41433-020-0770-y.

[164] M. Humayun, A. Santos, J.C. Altamirano, R. Ribeiro, R. Gonzalez, A. de la Rosa, J. Shih, C. Pang, F. Jiang, P. Calvillo, J. Huculak, J. Zimmerman, S. Caffey, Implantable MicroPump for Drug Delivery in Patients with Diabetic Macular Edema, Transl. Vis. Sci. Technol. 3 (2014) 5. doi:10.1167/tvst.3.6.5.

[165] S. Jiang, Y.L. Franco, Y. Zhou, J. Chen, Nanotechnology in retinal drug delivery, Int. J. Ophthalmol. 11 (2018) 1038–1044. doi:10.18240/ijo.2018.06.23.

[166] X. Huang, Y. Chau, Intravitreal nanoparticles for retinal delivery, Drug Discov. Today. 24 (2019) 1510–1523. doi:10.1016/j.drudis.2019.05.005.

[167] E. Himawan, P. Ekström, M. Buzgo, P. Gaillard, E. Stefánsson, V. Marigo, T. Loftsson, F. Paquet-Durand, Drug delivery to retinal photoreceptors, Drug Discov. Today. 24 (2019) 1637–1643. doi:10.1016/j.drudis.2019.03.004.

[168] J. Xu, Y. Wang, Y. Li, X. Yang, P. Zhang, H. Hou, Y. Shi, C. Song, Inhibitory Efficacy of Intravitreal Dexamethasone Acetate-Loaded PLGA Nanoparticles on Choroidal Neovascularization in a Laser-Induced Rat Model, J. Ocul. Pharmacol. Ther. 23 (2007) 527–540. doi:10.1089/jop.2007.0002.

[169] C.K. Pan, C. Durairaj, U.B. Kompella, O. Agwu, S.C.N. Oliver, H. Quiroz-Mercado, N. Mandava, J.L. Olson, Comparison of long-acting bevacizumab formulations in the treatment of choroidal neovascularization in a rat model, J. Ocul. Pharmacol. Ther. 27 (2011) 219–224. doi:10.1089/jop.2010.0158.

[170] U. Krach-Hansen, V.T.G. Chuang, M. Otagiri, Practical aspects of the ligand-binding and enzymatic properties of human serum albumin, Biol. Pharm. Bull. 25 (2002) 695–704. doi:10.1248/bpb.25.695.

[171] K.E. Kim, I. Jang, H. Moon, Y.J. Kim, J.W. Jeoung, K.H. Park, H. Kim, Neuroprotective effects of human serum albumin nanoparticles loaded with brimonidine on retinal ganglion cells in optic nerve crush model, Investig. Ophthalmol. Vis. Sci. 56 (2015) 5641–5649. doi:10.1167/iovs.15-16538.

[172] B.C. Ilochonwu, A. Urtti, W.E. Hennink, T. Vermonden, Intravitreal hydrogels for sustained release of therapeutic proteins, J. Control. Release. 326 (2020) 419–441.

doi:10.1016/j.jconrel.2020.07.031.

[173] D. Chang, K. Park, A. Famili, Hydrogels for sustained delivery of biologics to the back of the eye, Drug Discov. Today. 24 (2019) 1470–1482. doi:10.1016/j.drudis.2019.05.037.

[174] Y. Yu, L.C.M. Lau, A.C. Lo, Y. Chau, Injectable Chemically Crosslinked Hydrogel for the Controlled Release of Bevacizumab in Vitreous: A 6-Month In Vivo Study, Transl. Vis. Sci. Technol. 4 (2015) 5. doi:10.1167/tvst.4.2.5.

[175] S. Mukherjee, S. Ray, R.S. Thakur, Solid lipid nanoparticles: A modern formulation approach in drug delivery system, Indian J. Pharm. Sci. 71 (2009) 349–358. doi:10.4103/0250-474X.57282.

[176] P.S. Apaolaza, A. del Pozo-Rodríguez, M.A. Solinís, J.M. Rodríguez, U. Friedrich, J. Torrecilla, B.H.F. Weber, A. Rodríguez-Gascón, Structural recovery of the retina in a retinoschisin-deficient mouse after gene replacement therapy by solid lipid nanoparticles, Biomaterials. 90 (2016) 40–49. doi:10.1016/j.biomaterials.2016.03.004.

[177] J. Araújo, S. Nikolic, M.A. Egea, E.B. Souto, M.L. Garcia, Nanostructured lipid carriers for triamcinolone acetonide delivery to the posterior segment of the eye, Colloids Surfaces B Biointerfaces. 88 (2011) 150–157. doi:10.1016/j.colsurfb.2011.06.025.

[178] P. Simard, J.-C. Leroux, Pharmaceutical Nanotechnology pH-sensitive immunoliposomes specific to the CD33 cell surface antigen of leukemic cells, Int. J. Pharm. 381 (2009) 86–96. doi:10.1016/j.ijpharm.2009.05.013.

[179] J.G. Christie, U.B. Kompella, Ophthalmic light sensitive nanocarrier systems, Drug Discov. Today. 13 (2008) 124–134. doi:10.1016/j.drudis.2007.12.005.

[180] V.A.N. Huu, J. Luo, J. Zhu, J. Zhu, S. Patel, A. Boone, E. Mahmoud, C. McFearin, J. Olejniczak, C. De Gracia Lux, J. Lux, N. Fomina, M. Huynh, K. Zhang, A. Almutairi, Light-responsive nanoparticle depot to control release of a small molecule angiogenesis inhibitor in the posterior segment of the eye, J. Control. Release. 200 (2015) 71–77. doi:10.1016/j.jconrel.2015.01.001.

[181] J.S. Basuki, F. Qie, X. Mulet, R. Suryadinata, A. V. Vashi, Y.Y. Peng, L. Li, X. Hao, T. Tan, T.C. Hughes, Photo-Modulated Therapeutic Protein Release from a Hydrogel Depot Using Visible Light, Angew. Chemie - Int. Ed. 56 (2017) 966–971. doi:10.1002/anie.201610618.

[182] W.L.L. Suen, Y. Chau, Specific uptake of folate-decorated triamcinolone-encapsulating nanoparticles by retinal pigment epithelium cells enhances and prolongs antiangiogenic activity, J. Control. Release. 167 (2013) 21–28. doi:10.1016/j.jconrel.2013.01.004.

[183] O.K. Kari, S. Tavakoli, P. Parkkila, S. Baan, R. Savolainen, T. Ruoslahti, N.G. Johansson, J. Ndika, H. Alenius, T. Viitala, A. Urtti, T. Lajunen, Light-Activated Liposomes Coated with Hyaluronic Acid as a Potential Drug Delivery System, Pharmaceutics. 12 (2020) 763. doi:10.3390/pharmaceutics12080763.

[184] S. Nishina, A. Hirakata, T. Hida, H. Sawa, N. Azuma, CD44 expression in the developing human retina, Graefe's Arch. Clin. Exp. Ophthalmol. 235 (1997) 92–96.

doi:10.1007/BF00941736.

[185] L. Gan, J. Wang, Y. Zhao, D. Chen, C. Zhu, J. Liu, Y. Gan, Hyaluronan-modified core-shell liponanoparticles targeting CD44-positive retinal pigment epithelium cells via intravitreal injection, Biomaterials. 34 (2013) 5978–5987. doi:10.1016/j.biomaterials.2013.04.035.

[186] D. Huang, Y.S. Chen, C.R. Green, I.D. Rupenthal, Hyaluronic acid coated albumin nanoparticles for targeted peptide delivery in the treatment of retinal ischaemia, Biomaterials. 168 (2018) 10–23. doi:10.1016/j.biomaterials.2018.03.034.

[187] M. Giannaccini, L. Pedicini, G. De Matienzo, F. Chiellini, L. Dente, V. Raffa, Magnetic nanoparticles: A strategy to target the choroidal layer in the posterior segment of the eye, Sci. Rep. 7 (2017) 1–11. doi:10.1038/srep43092.

[188] A.D. Bangham, M.M. Standish, J.C. Watkins, Diffusion of univalent ions across the lamellae of swollen phospholipids, J. Mol. Biol. 13 (1965) 238–252. doi:10.1016/S0022-2836(65)80093-6.

[189] T.M. Allen, P.R. Cullis, Liposomal drug delivery systems: From concept to clinical applications ☆, Adv. Drug Deliv. Rev. 65 (2013) 36–48. doi:10.1016/j.addr.2012.09.037.

[190] A. Jesorka, O. Orwar, Liposomes: Technologies and analytical applications, Annu. Rev. Anal. Chem. 1 (2008) 801–832. doi:10.1146/annurev.anchem.1.031207.112747.

[191] A. Bochot, E. Fattal, Liposomes for intravitreal drug delivery: A state of the art, J. Control. Release. 161 (2012) 628–634. doi:10.1016/j.jconrel.2012.01.019.

[192] G.P. Mishra, M. Bagui, V. Tamboli, A.K. Mitra, Recent Applications of Liposomes in Ophthalmic Drug Delivery, J. Drug Deliv. 2011 (2011) 14. doi:10.1155/2011/863734.

[193] T.F. Martens, K. Peynshaert, T.L. Nascimento, E. Fattal, M. Karlstetter, T. Langmann, S. Picaud, J. Demeester, S.C. De Smedt, K. Remaut, K. Braeckmans, Effect of hyaluronic acid-binding to lipoplexes on intravitreal drug delivery for retinal gene therapy, Eur. J. Pharm. Sci. 103 (2017) 27–35. doi:10.1016/j.ejps.2017.02.027.

[194] R. Zhang, R. He, J. Qian, J. Guo, K. Xue, Y.F. Yuan, Treatment of experimental autoimmune uveoretinitis with intravitreal injection of tacrolimus (FK506) encapsulated in liposomes, Investig. Ophthalmol. Vis. Sci. 51 (2010) 3575–3582. doi:10.1167/iovs.09-4373.

[195] B. Wiechens, J.B. Grammer, U. Johannsen, U. Pleyer, J. Hedderich, G.I.W. Duncker, Experimental Intravitreal Application of Ciprofloxacin in Rabbits, Ophthalmologica. 213 (1999) 120–128. doi:10.1159/000027404.

[196] J.P. Cannon, R. Fiscella, S. Pattharachayakul, K.W. Garey, F. De Alba, S. Piscitelli, D.P. Edward, L.H. Danziger, Comparative toxicity and concentrations of intravitreal amphotericin B formulations in a rabbit model, Investig. Ophthalmol. Vis. Sci. 44 (2003) 2112–2117. doi:10.1167/iovs.02-1020.

[197] M. Abrishami, S. Zarei-Ghanavati, D. Soroush, M. Rouhbakhsh, M.R. Jaafari, B.

Malaekeh-Nikouei, Preparation, characterization, and in vivo evaluation of nanoliposomes-encapsulated bevacizumab (avastin) for intravitreal administration, Retina. 29 (2009) 699–703. doi:10.1097/IAE.0b013e3181a2f42a.

[198] S. Kawakami, A. Harada, K. Sakanaka, K. Nishida, J. Nakamura, T. Sakaeda, N. Ichikawa, M. Nakashima, H. Sasaki, In vivo gene transfection via intravitreal injection of cationic liposome/plasmid DNA complexes in rabbits, Int. J. Pharm. 278 (2004) 255–262. doi:10.1016/j.ijpharm.2004.03.013.

[199] H.A. Liu, Y. ling Liu, Z. zhong Ma, J. cheng Wang, Q. Zhang, A lipid nanoparticle system improves siRNA efficacy in RPE cells and a laser-induced murine CNV model, Investig. Ophthalmol. Vis. Sci. 52 (2011) 4789–4794. doi:10.1167/iovs.10-5891.

[200] Y. Malam, M. Loizidou, A.M. Seifalian, Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer, Trends Pharmacol. Sci. 30 (2009) 592–599. doi:10.1016/j.tips.2009.08.004.

[201] J. lin Wang, Y. ling Liu, Y. Li, W. bing Dai, Z. ming Guo, Z. hui Wang, Q. Zhang, EphA2 targeted doxorubicin stealth liposomes as a therapy system for choroidal neovascularization in rats, Investig. Ophthalmol. Vis. Sci. 53 (2012) 7348–7357. doi:10.1167/iovs.12-9955.

[202] M.F. Zuluaga, C. Mailhos, G. Robinson, D.T. Shima, R. Gurny, N. Lange, Synergies of VEGF inhibition and photodynamic therapy in the treatment of age-related macular degeneration, Investig. Ophthalmol. Vis. Sci. 48 (2007) 1767–1772. doi:10.1167/iovs.06-1224.

[203] T. Lajunen, L.S. Kontturi, L. Viitala, M. Manna, O. Cramariuc, T. Róg, A. Bunker, T. Laaksonen, T. Viitala, L. Murtomäki, A. Urtti, Indocyanine Green-Loaded Liposomes for Light-Triggered Drug Release, Mol. Pharm. 13 (2016) 2095–2107. doi:10.1021/acs.molpharmaceut.6b00207.

[204] T. Lajunen, R. Nurmi, L.-S. Kontturi, L. Viitala, M. Yliperttula, L. Murtomäki, A. Urtti, Light activated liposomes: Functionality and prospects in ocular drug delivery, J. Control. Release. 224 (2016) 157–166.

[205] L. Peeters, N.N. Sanders, K. Braeckmans, K. Boussery, J. Van De Voorde, S.C. De Smedt, J. Demeester, Vitreous: A barrier to nonviral ocular gene therapy, Investig. Ophthalmol. Vis. Sci. 46 (2005) 3553–3561. doi:10.1167/iovs.05-0165.

[206] Q. Xu, N.J. Boylan, J.S. Suk, Y.Y. Wang, E.A. Nance, J.C. Yang, P.J. McDonnell, R.A. Cone, E.J. Duh, J. Hanes, Nanoparticle diffusion in, and microrheology of, the bovine vitreous ex vivo, J. Control. Release. 167 (2013) 76–84. doi:10.1016/j.jconrel.2013.01.018.

[207] T.F. Martens, D. Vercauteren, K. Forier, H. Deschout, K. Remaut, R. Paesen, M. Ameloot, J.F. Engbersen, J. Demeester, S.C. De Smedt, K. Braeckmans, Measuring the intravitreal mobility of nanomedicines with single-particle tracking microscopy, Nanomedicine. 8 (2013) 1955–1968. doi:10.2217/nnm.12.202.

[208] B.T. Käsdorf, F. Arends, O. Lieleg, Diffusion Regulation in the Vitreous Humor, Biophys. J. 109 (2015) 2171–2181. doi:10.1016/j.bpj.2015.10.002.

[209] A.Z. Eriksen, J. Brewer, T.L. Andresen, A.J. Urquhart, The diffusion dynamics of

PEGylated liposomes in the intact vitreous of the ex vivo porcine eye: A fluorescence correlation spectroscopy and biodistribution study, Int. J. Pharm. 522 (2017) 90–97. doi:10.1016/j.ijpharm.2017.03.003.

[210] G.P. Ochoa, J.Z. Sesma, M.A. Díez, A. Díaz-Tahoces, M. Avilés-Trigeros, S. Grijalvo, R. Eritja, E. Fernández, J.L. Pedraz, A novel formulation based on 2,3-di(tetradecyloxy)propan-1-amine cationic lipid combined with polysorbate 80 for efficient gene delivery to the retina, Pharm. Res. 31 (2014) 1665–1675. doi:10.1007/s11095-013-1271-5.

[211] E. Ojeda, G. Puras, M. Agirre, J. Zarate, S. Grijalvo, R. Eritja, G. Martinez-Navarrete, C. Soto-Sánchez, A. Diaz-Tahoces, M. Aviles-Trigueros, E. Fernández, J.L. Pedraz, The influence of the polar head-group of synthetic cationic lipids on the transfection efficiency mediated by niosomes in rat retina and brain, Biomaterials. 77 (2016) 267–279. doi:10.1016/j.biomaterials.2015.11.017.

[212] J. Mosinger Ogilvie, J.D. Speck, J.M. Lett, T.T. Fleming, A reliable method for organ culture of neonatal mouse retina with long- term survival, J. Neurosci. Methods. 87 (1999) 57–65. doi:10.1016/S0165-0270(98)00157-5.

[213] J. Pang, M. Cheng, D. Stevenson, M.D. Trousdale, C.K. Dorey, J.C. Blanks, Adenoviral-mediated gene transfer to retinal explants during development and degeneration, Exp. Eye Res. 79 (2004) 189–201. doi:10.1016/j.exer.2004.03.010.

[214] E. Söderstjerna, P. Bauer, T. Cedervall, H. Abdshill, F. Johansson, U.E. Johansson, Silver and Gold Nanoparticles Exposure to In Vitro Cultured Retina – Studies on Nanoparticle Internalization, Apoptosis, Oxidative Stress, Glial- and Microglial Activity, PLoS One. 9 (2014) e105359. doi:10.1371/journal.pone.0105359.

[215] M. Fradot, V. Busskamp, V. Forster, T. Cronin, T. Léveillard, J. Bennett, J.A. Sahel, B. Roska, S. Picaud, Gene therapy in ophthalmology: Validation on cultured retinal cells and explants from postmortem human eyes, Hum. Gene Ther. 22 (2011) 587–593. doi:10.1089/hum.2010.157.

[216] J. Devoldere, K. Peynshaert, H. Dewitte, C. Vanhove, L. De Groef, L. Moons, S.Y. Özcan, D. Dalkara, S.C. De Smedt, K. Remaut, Non-viral delivery of chemically modified mRNA to the retina: Subretinal versus intravitreal administration, J. Control. Release. 307 (2019) 315–330. doi:10.1016/j.jconrel.2019.06.042.

[217] J. Valdés, L. Trachsel-Moncho, A. Sahaboglu, D. Trifunović, M. Miranda, M. Ueffing, F. Paquet-Durand, O. Schmachtenberg, Organotypic retinal explant cultures as in vitro alternative for diabetic retinopathy studies, ALTEX. 33 (2016) 459–464. doi:10.14573/altex.1603111.

[218] T.L. Jackson, R.J. Antcliff, J. Hillenkamp, J. Marshall, Human retinal molecular weight exclusion limit and estimate of species variation, Investig. Ophthalmol. Vis. Sci. 44 (2003) 2141–2146. doi:10.1167/iovs.02-1027.

[219] Y. Tao, X. Li, Y. Jiang, X. Bai, B. Wu, J. Dong, Diffusion of Macromolecule Through Retina After Experimental Branch Retinal Vein Occlusion and Estimate of Intraretinal Barrier, Curr. Drug Metab. 8 (2007) 151–156. doi:10.2174/138920007779815968.

[220] M. Kimura, M. Araie, S. Koyano, Movement of carboxyfluorescein across retinal

pigment epithelium-choroid, Exp. Eye Res. 63 (1996) 51–56. doi:10.1006/exer.1996.0090.

[221] L. Pitkänen, V.P. Ranta, H. Moilanen, A. Urtti, Permeability of retinal pigment epithelium: Effects of permeant molecular weight and lipophilicity, Investig. Ophthalmol. Vis. Sci. 46 (2005) 641–646. doi:10.1167/iovs.04-1051.

[222] L. Hellinen, H. Hongisto, E. Ramsay, K. Kaarniranta, K.S. Vellonen, H. Skottman, M. Ruponen, Drug flux across RPE cell models: The hunt for an appropriate outer blood–retinal barrier model for use in early drug discovery, Pharmaceutics. 12 (2020). doi:10.3390/pharmaceutics12020176.

[223] S.R.S.S.E.R.E.H.F.E.U.B. Kompella, Particle Surface Functionalization for Enhanced Nanoparticle Delivery to the Anterior and the Posterior Segments of the Eye, Invest Ophthalmol Vis Sci. 48 (2007). https://iovs.arvojournals.org/article.aspx?articleid=2388469 (accessed December 16, 2020).

[224] P.G.M. HOFF, Retinal Function in an Isolated, Perfused Mammalian Eye | IOVS | ARVO Journals, Invest. Ophthalmol. Vis. Sci. 9 (1970) 388–399. https://iovs.arvojournals.org/article.aspx?articleid=2166902 (accessed December 17, 2020).

[225] M.J. Koeberle, P.M. Hughes, G.G. Skellern, C.G. Wilson, Pharmacokinetics and disposition of memantine in the arterially perfused bovine eye, Pharm. Res. 23 (2006) 2781–2798. doi:10.1007/s11095-006-9106-2.

[226] J. Mains, L.E. Tan, C. Wilson, A. Urquhart, A pharmacokinetic study of a combination of beta adrenoreceptor antagonists - In the isolated perfused ovine eye, in: Eur. J. Pharm. Biopharm., Elsevier, 2012: pp. 393–401. doi:10.1016/j.ejpb.2011.11.006.

[227] J. Mains, C.G. Wilson, A. Urquhart, ToF-SIMS analysis of dexamethasone distribution in the isolated perfused eye, Investig. Ophthalmol. Vis. Sci. 52 (2011) 8413–8419. doi:10.1167/iovs.11-8199.

[228] E.M. Abarca, J.H. Salmon, B.C. Gilger, Effect of choroidal perfusion on ocular tissue distribution after intravitreal or suprachoroidal injection in an arterially perfused ex vivo pig eye model, J. Ocul. Pharmacol. Ther. 29 (2013) 715–722. doi:10.1089/jop.2013.0063.

[229] M. Jost, C. Maillard, J. Lecomte, V. Lambert, M. Tjwa, P. Blaise, M.L.A. Gonzalez, K. Bajou, S. Blacher, P. Motte, C. Humblet, M.P. Defresne, M. Thiry, F. Frankenne, A. Gothot, P. Carmeliet, J.M. Rakic, J.M. Foidart, A. Noël, Tumoral and choroidal vascularization: Differential cellular mechanisms involving plasminogen activator inhibitor type I, Am. J. Pathol. 171 (2007) 1369–1380. doi:10.2353/ajpath.2007.070074.

[230] A. Noël, M. Jost, V. Lambert, J. Lecomte, J.M. Rakic, Anti-angiogenic therapy of exudative age-related macular degeneration: current progress and emerging concepts, Trends Mol. Med. 13 (2007) 345–352. doi:10.1016/j.molmed.2007.06.005.

[231] K. Shinjiro, F. Mizuki, K. Hitoshi, Y. Sumino, K. Ikuko, A Quantitative Assay for

Angiogenesis of Cultured Choroidal Tissues in Streptozotocin-Diabetic Wistar and Spontaneously Diabetic GK Rats, Jpn. J. Pharmacol. 78 (1998) 471–478. doi:10.1254/jjp.78.471.

[232] Z. Shao, M. Friedlander, C.G. Hurst, Z. Cui, D.T. Pei, L.P. Evans, A.M. Juan, H. Tahir, F. Duhamel, J. Chen, P. Sapieha, S. Chemtob, J.-S. Joyal, L.E.H. Smith, Choroid Sprouting Assay: An Ex Vivo Model of Microvascular Angiogenesis, PLoS One. 8 (2013) e69552. doi:10.1371/journal.pone.0069552.

[233] C. Nie, M.N. Zhang, H.W. Zhao, T.D. Olsen, K. Jackman, L.N. Hu, W.P. Ma, X.F. Chen, J. Wang, Y. Zhang, T.S. Gao, H. Uehara, B.K. Ambati, L. Luo, Correlation of in vivo and in vitro methods in measuring choroidal vascularization volumes using a subretinal injection induced choroidal neovascularization model, Chin. Med. J. (Engl). 128 (2015) 1516–1522. doi:10.4103/0366-6999.157681.

[234] M.E. Pennesi, M. Neuringer, R.J. Courtney, Animal models of age related macular degeneration, Mol. Aspects Med. 33 (2012) 487–509. doi:10.1016/j.mam.2012.06.003.

[235] H.E. Grossniklaus, S.J. Kang, L. Berglin, Animal models of choroidal and retinal neovascularization, Prog. Retin. Eye Res. 29 (2010) 500–519. doi:10.1016/j.preteyeres.2010.05.003.

[236] V. Lambert, J. Lecomte, S. Hansen, S. Blacher, M.L.A. Gonzalez, I. Struman, N.E. Sounni, E. Rozet, P. De Tullio, J.M. Foidart, J.M. Rakic, A. Noel, Laser-induced choroidal neovascularization model to study age-related macular degeneration in mice, Nat. Protoc. 8 (2013) 2197–2211. doi:10.1038/nprot.2013.135.

[237] N. Sengupta, A. Afzal, S. Caballero, K.H. Chang, L.C. Shaw, J.J. Pang, V.C. Bond, I. Bhutto, T. Baba, G.A. Lutty, M.B. Grant, Paracrine modulation of CXCR4 by IGF-1 and VEGF: Implications for choroidal neovascularization, Investig. Ophthalmol. Vis. Sci. 51 (2010) 2697–2704. doi:10.1167/iovs.09-4137.

[238] L. Luo, X. Zhang, Y. Hirano, P. Tyagi, P. Barabás, H. Uehara, T.R. Miya, N. Singh, B. Archer, Y. Qazi, K. Jackman, S.K. Das, T. Olsen, S.R. Chennamaneni, B.C. Stagg, F. Ahmed, L. Emerson, K. Zygmunt, R. Whitaker, C. Mamalis, W. Huang, G. Gao, S.P. Srinivas, D. Krizaj, J. Baffi, J. Ambati, U.B. Kompella, B.K. Ambati, Targeted intraceptor nanoparticle therapy reduces angiogenesis and fibrosis in primate and murine macular degeneration, ACS Nano. 7 (2013) 3264–3275. doi:10.1021/nn305958y.

[239] J.Z. Cui, H. Kimura, C. Spee, G. Thumann, D.R. Hinton, S.J. Ryan, Natural history of choroidal neovascularization induced by vascular endothelial growth factor in the primate, Graefe's Arch. Clin. Exp. Ophthalmol. 238 (2000) 326–333. doi:10.1007/s004170050360.

[240] J.S. Heier, D.M. Brown, V. Chong, J.F. Korobelnik, P.K. Kaiser, Q.D. Nguyen, B. Kirchhof, A. Ho, Y. Ogura, G.D. Yancopoulos, N. Stahl, R. Vitti, A.J. Berliner, Y. Soo, M. Anderesi, G. Groetzbach, B. Sommerauer, R. Sandbrink, C. Simader, U. Schmidt-Erfurth, Intravitreal aflibercept (VEGF trap-eye) in wet age-related macular degeneration, Ophthalmology. 119 (2012) 2537–2548. doi:10.1016/j.ophtha.2012.09.006.

[241] P.J. Rosenfeld, R.M. Rich, G.A. Lalwani, Ranibizumab: Phase III Clinical Trial

Results, Ophthalmol. Clin. North Am. 19 (2006) 361–372. doi:10.1016/j.ohc.2006.05.009.

[242] I. Sanchez, R. Martin, F. Ussa, I. Fernandez-Bueno, The parameters of the porcine eyeball, Graefe's Arch. Clin. Exp. Ophthalmol. 249 (2011) 475–482. doi:10.1007/s00417-011-1617-9.

[243] K.E. Swindle, P.D. Hamilton, N. Ravi, In situ formation of hydrogels as vitreous substitutes: Viscoelastic comparison to porcine vitreous, J. Biomed. Mater. Res. - Part A. 87 (2008) 656–665. doi:10.1002/jbm.a.31769.

[244] B. Lee, M. Litt, G. Buchsbaum, Rheology of the vitreous body. Part I: Viscoelasticity of human vitreous, Biorheology. 29 (1992) 521–533. doi:10.3233/BIR-1992-295-612.

[245] B. Lee, M. Litt, G. Buchsbaum, Rheology of the vitreous body: Part 2. Viscoelasticity of bovine and porcine vitreous, Biorheology. 31 (1994) 327–338. doi:10.3233/BIR-1994-31403.

[246] R. Vedadghavami, UCLA UCLA Electronic Theses and Dissertations Title Rheology of Vitreous Gel, 2015. https://escholarship.org/uc/item/19d4g3zd (accessed January 13, 2021).

[247] J. Sebag, Age-related changes in human vitreous structure, Graefes Arch. Clin. Exp. Ophthalmol. 225 (1987) 89–93. doi:10.1007/BF02160337.

[248] B. Lee, M. Litt, G. Buchsbaum, Rheology of the vitreous body: Part 3. Concentration of electrolytes, collagen and hyaluronic acid, Biorheology. 31 (1994) 339–351. doi:10.3233/BIR-1994-31404.

[249] N. Ruthardt, D.C. Lamb, C. Bräuchle, Single-particle tracking as a quantitative microscopy-based approach to unravel cell entry mechanisms of viruses and pharmaceutical nanoparticles, Mol. Ther. 19 (2011) 1199–1211. doi:10.1038/mt.2011.102.

[250] X. Michalet, Mean Square Displacement Analysis of Single-Particle Trajectories with Localization Error: Brownian Motion in Isotropic Medium, n.d. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3055791/pdf/nihms273605.pdf (accessed October 17, 2018).

[251] E.I. Altiok, S. Browne, E. Khuc, E.P. Moran, F. Qiu, K. Zhou, J.L. Santiago-Ortiz, J.X. Ma, M.F. Chan, K.E. Healy, sFlt multivalent conjugates inhibit angiogenesis and improve half-life in vivo, PLoS One. 11 (2016). doi:10.1371/journal.pone.0155990.

[252] D. Huang, Y.S. Chen, C.R. Green, I.D. Rupenthal, Hyaluronic acid coated albumin nanoparticles for targeted peptide delivery in the treatment of retinal ischaemia, Biomaterials. 168 (2018) 10–23. doi:10.1016/j.biomaterials.2018.03.034.

[253] C.D. Walkey, W.C.W. Chan, Understanding and controlling the interaction of nanomaterials with proteins in a physiological environment, Chem. Soc. Rev. 41 (2012) 2780–2799. doi:10.1039/c1cs15233e.

[254] A. Amici, G. Caracciolo, L. Digiacomo, V. Gambini, C. Marchini, M. Tilio, A.L. Capriotti, V. Colapicchioni, R. Matassa, G. Familiari, S. Palchetti, D. Pozzi, M.

Mahmoudi, A. Laganà, In vivo protein corona patterns of lipid nanoparticles, RSC Adv. 7 (2017) 1137–1145. doi:10.1039/c6ra25493d.

[255] Fda, Cder, Nanotechnology—Over a Decade of Progress and Innovation: A Report by the U.S. Food and Drug Administration, 2020. https://www.nano.gov/nanotech-101/what (accessed January 14, 2021).

[256] S.R. D'Mello, C.N. Cruz, M.L. Chen, M. Kapoor, S.L. Lee, K.M. Tyner, The evolving landscape of drug products containing nanomaterials in the United States, Nat. Nanotechnol. 12 (2017) 523–529. doi:10.1038/nnano.2017.67.

[257] Fda, Cder, Liposome Drug Products Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation Guidance for Industry, 2018. http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm (accessed January 14, 2021).

[258] H. Koo, H. Moon, H. Han, J.H. Na, M.S. Huh, J.H. Park, S.J. Woo, K.H. Park, I. Chan Kwon, K. Kim, H. Kim, The movement of self-assembled amphiphilic polymeric nanoparticles in the vitreous and retina after intravitreal injection, Biomaterials. 33 (2012) 3485–3493. doi:10.1016/j.biomaterials.2012.01.030.

[259] A. Popov, E.M. Enlow, H. Chen, Compositions and methods for ophthalmic and/or other applications, 2017. https://patents.google.com/patent/US9827191B2/en (accessed October 15, 2020).

[260] S. Vaz-Pereira, T. Morais-Sarmento, R. Esteves Marques, Optical coherence tomography features of neovascularization in proliferative diabetic retinopathy: A systematic review, Int. J. Retin. Vitr. 6 (2020) 26. doi:10.1186/s40942-020-00230-3.

[261] R.J. Motzer, B. Escudier, A. Gannon, R.A. Figlin, Sunitinib: Ten Years of Successful Clinical Use and Study in Advanced Renal Cell Carcinoma, Oncologist. 22 (2017) 41–52. doi:10.1634/theoncologist.2016-0197.

[262] R.J. Motzer, B.I. Rini, R.M. Bukowski, B.D. Curti, D.J. George, G.R. Hudes, B.G. Redman, K.A. Margolin, J.R. Merchan, G. Wilding, M.S. Ginsberg, J. Bacik, S.T. Kim, C.M. Baum, M.D. Michaelson, Sunitinib in patients with metastatic renal cell carcinoma, J. Am. Med. Assoc. 295 (2006) 2516–2524. doi:10.1001/jama.295.21.2516.

[263] R. Roskoski, Sunitinib: A VEGF and PDGF receptor protein kinase and angiogenesis inhibitor, Biochem. Biophys. Res. Commun. 356 (2007) 323–328. doi:10.1016/j.bbrc.2007.02.156.

[264] H. Tsujinaka, J. Fu, J. Shen, Y. Yu, Z. Hafiz, J. Kays, D. McKenzie, D. Cardona, D. Culp, W. Peterson, B.C. Gilger, C.S. Crean, J.Z. Zhang, Y. Kanan, W. Yu, J.L. Cleland, M. Yang, J. Hanes, P.A. Campochiaro, Sustained treatment of retinal vascular diseases with self-aggregating sunitinib microparticles, Nat. Commun. 11 (2020). doi:10.1038/s41467-020-14340-x.

[265] H. Takahashi, R. Obata, Y. Tamaki, A novel vascular endothelial growth factor receptor 2 inhibitor, SU11248, suppresses choroidal neovascularization in vivo, J. Ocul. Pharmacol. Ther. 22 (2006) 213–218. doi:10.1089/jop.2006.22.213.

[266] S. Ebrahim, G.A. Peyman, P.J. Lee, Applications of liposomes in ophthalmology,

Surv. Ophthalmol. 50 (2005) 167–182. doi:10.1016/j.survophthal.2004.12.006.

[267] Crosslinked Hyaluronic Acid With Liposomes and Crocin in Dry Eye - Full Text View - ClinicalTrials.gov,(n.d.).https://clinicaltrials.gov/ct2/show/NCT03617315?term=liposomal+eye+drop&cond=Dry+Eye&draw=2&rank=4 (accessed January 27, 2021).

[268] J.H.C.F.B.E. Astafieva, Irina, Systems for sustained intraocular delivery of low solubility compounds from a port delivery system implant related, applications, 2014, (accessed December 31, 2020)

[269] A.K. Rimpelä, M. Schmitt, S. Latonen, M. Hagström, M. Antopolsky, J.A. Manzanares, H. Kidron, A. Urtti, Drug distribution to retinal pigment epithelium: Studies on melanin binding, cellular kinetics, and single photon emission computed tomography/computed tomography imaging, Mol. Pharm. 13 (2016) 2977–2986. doi:10.1021/acs.molpharmaceut.5b00787.

[270] R. Varela-Fernández, V. Díaz-Tomé, A. Luaces-Rodríguez, A. Conde-Penedo, X. García-Otero, A. Luzardo-álvarez, A. Fernández-Ferreiro, F.J. Otero-Espinar, Drug delivery to the posterior segment of the eye: Biopharmaceutic and pharmacokinetic considerations, Pharmaceutics. 12 (2020). doi:10.3390/pharmaceutics12030269.

[271] P. Jakubiak, C. Cantrill, A. Urtti, A. Urtti, R. Alvarez-Sánchez, Establishment of an in Vitro-In Vivo Correlation for Melanin Binding and the Extension of the Ocular Half-Life of Small-Molecule Drugs, Mol. Pharm. 16 (2019) 4890–4901. doi:10.1021/acs.molpharmaceut.9b00769.

[272] J.E. Grunwald, E. Daniel, J. Huang, G.S. Ying, M.G. Maguire, C.A. Toth, G.J. Jaffe, S.L. Fine, B. Blodi, M.L. Klein, A.A. Martin, S.A. Hagstrom, D.F. Martin, Risk of geographic atrophy in the comparison of age-related macular degeneration treatments trials, Ophthalmology. 121 (2014) 150–161. doi:10.1016/j.ophtha.2013.08.015.

[273] Y. Niwa, M. Kakinoki, T. Sawada, X. Wang, M. Ohji, Ranibizumab and aflibercept: Intraocular pharmacokinetics and their effects on aqueous VEGF level in vitrectomized and nonvitrectomized macaque eyes, Investig. Ophthalmol. Vis. Sci. 56 (2015) 6501–6505. doi:10.1167/iovs.15-17279.

[274] M. Edington, J. Connolly, N.V. Chong, Pharmacokinetics of intravitreal anti-VEGF drugs in vitrectomized versus non-vitrectomized eyes, Expert Opin. Drug Metab. Toxicol. 13 (2017) 1217–1224. doi:10.1080/17425255.2017.1404987.

[275] J. Xu, J.J. Heys, V.H. Barocas, T.W. Randolph, Permeability and diffusion in vitreous humor: Implications for drug delivery, Pharm. Res. 17 (2000) 664–669. doi:10.1023/A:1007517912927.

[276] D. Huang, Y.-S. Chen, Q. Xu, J. Hanes, I.D. Rupenthal, Effects of enzymatic degradation on dynamic mechanical properties of the vitreous and intravitreal nanoparticle mobility, Eur. J. Pharm. Sci. 118 (2018) 124–133. doi:10.1016/j.ejps.2018.03.023.

[277] M. To, A. Goz, L. Camenzind, P. Oertle, J. Candiello, M. Sullivan, P.B. Henrich, M. Loparic, F. Safi, A. Eller, W. Halfter, Diabetes-induced morphological, biomechanical, and compositional changes in ocular basement membranes, Exp.

Eye Res. 116 (2013) 298–307. doi:10.1016/j.exer.2013.09.011.

[278] R. Ridolfo, S. Tavakoli, V. Junnuthula, D.S. Williams, A. Urtti, J.C.M. van Hest, Exploring the impact of morphology on the properties of biodegradable nanoparticles and their diffusion in complex biological medium, Biomacromolecules. (2020). doi:10.1021/acs.biomac.0c00726.

[279] T. Zhang, C.D. Xiang, D. Gale, S. Carreiro, E.Y. Wu, E.Y. Zhang, Drug transporter and cytochrome P450 mRNA expression in human ocular barriers: Implications for ocular drug disposition, Drug Metab. Dispos. 36 (2008) 1300–1307. doi:10.1124/dmd.108.021121.